The role of JAZs and GABA in Plant Immune Response

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I certify that all material in this dissertation which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Jasmonic acid (JA) is a phytohormone with diverse roles in plant; development, growth and immune response. Jasmonic acid ZIM domain repressor (JAZ) proteins are important negative regulators of JA signalling. It has been shown that JAZs play an important role in the immune response of *Arabidopsis thaliana* to *Pseudomonas syringae,* as the pathogen seeks to exploit the JA signalling pathway to weaken *A. thaliana*'s immune response. While JA signalling is vital for immune responses to necrotrophic pathogens such as *Botrytis cinerea*, it is antagonistic to salicylic acid signalling necessary for immune response to biotrophic pathogens such as *P. syringae*. Through the use of a yeast two hybrid system we sought to identify novel interactors of JAZ proteins to further map the JAZ interactome during pathogen infection and to show that the JAZ proteins interactomes change during pathogen induced stress. We screened cDNA libraries created from RNA transcripts harvested from *A. thaliana* during *P. syringae* infection, with a JAZ5 containing bait vector. We identified two putative, novel interactors of JAZ5; GSTF10 and HSC70-1 both of which have roles in plant defense and affect salicylic acid signalling. We also investigated the effect of JAZs on defence against necrotrophs by infecting *At-jaz* mutants with *B. cinerea.* We identified *At-jaz5/7* and *At-jaz7/10* as more susceptible to *B. cinerea* infection than wild type plants and hypothesized that this may be due to the removal of JAZ splice variants responsible for preventing runaway JA responses. Additionally we sought to further previous work on the role of γ-aminobutyric acid (GABA) in *A. thaliana* during pathogen infection. GABA has been shown to act as a source of nutrition for *P. syringae* but can repress pathogenesis genes. Using mass spectrometry we measured the amino acid content of *A. thaliana* amino acid transporter mutants inoculated with *P. syringae* as well as investigating the effect of GABA concentrations on *P. syringae* growth. We found that *At-at5g41800/gat-1* plants inoculated with *P. syringae* and 10mM GABA exhibited significantly lowered *P. syringae* growth compared to inoculations of *P. syringae* alone.

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Contents

Definitions

2ODD	2-oxoglutarate-dependent dioxygenases
AAAP1-6	Amino acid permease 1-6
AAC	
AAO3	Aldehyde oxidase 3
AAO4	Aldehyde oxidase 4
ABA	Abscisic Acid
ABA2	Alcohol dehydrogenase 2
ABF	Abscisic acid responsive promoter element binding factor
ABF4	Abscisic acid responsive promoter element binding factor 4
ABI5/4	Abscisic acid insensitive 4/5
ABRE	Abscisic acid responsive promoter elements
ACC	1-aminocyclopropane-1-carboxylic acid
Acryl/bis	Acrylamide/Bis-acrylamide
ACX	Acyl coenzyme A oxidase
AFP	Absisic acid insensitive five binding protein
ALC	Alcatraz
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ATP	Adenosine triphosphate
Avr	Avirulence protein
bHLH	Basic helix-loop-helix protein
CC	Coiled coil domain
CC-NB-LRR	Coiled coil nucleotide binding leucine rich repeat domain
CFA	coronafacic acid
CHLH	Chelatase H subunit
CM	Chorismate mutase
CMA	Coronamic acid
COR	Coronatine
COR1	Coronatine induced protein 1

CPS	*Ent*-copalyl diphosphate synthase
CTR1	Constitutive triple response 1
CUL3	Cullin 3
DAD1	Defective in anther dehiscence 1
DDO/X/A	Yeast Potato Dextrose Adenine media containing aureobasidin A and 5-Bromo-4-Chloro-3-indolyl α-D-galactopyranoside (X-α-Gal) that does not contain leucine or tryptophan
DinorOPDA	Dinor-oxophytodienoic acid
DMSO	Dimethyl sulfoxide
EAR	Ethylene-responsive element binding factor-associated amphiphilic repression motif
EBF1-4	Ethylene insensitive 3 binding F-box protein 1-4
EDS1	Enhanced disease susceptibilitiy 1
EFR	Elongation factor Tu receptor
EGL3	Enhancer of GLABARA3
EIL1	Ethylene insensitive 3 like protein 1
EIN2/3/4	Ethylene insensitive 2-4
ER	Endoplasmic reticulum
ERF1/5/6	Ethylene responsive factor 1/5/6
ERS1/2	Ethylene response sensor 1
Et	Ethylene
ETI	Effector triggered immunity
ETP1/2	Ethylene insensitive 2 targeting protein
ETR1/2	Ethylene response 1/2
ETS	Effector triggered susceptibility
FLS2	Flagellin sensing 2
G-Box	CACGTG nucleotide seqeuence
GA	Gibberellin
GABA	γ-aminobutyric acid
GABAT	γ-aminobutyric acid transaminase

GABP	γ-aminobutyric acid permease
GAD	Glutamate decarboxylase
GAD1/GAD2	Glutamate decarboxylase 1/2
GAI1	Gibberellin insensitive 1
GAL4 AD	GAL4 activation domain
GAL4 BD	GAL4 binding domain
GDP	Guanosine diphosphate
GGPP	*Trans*-geranylgeranyldiphosphate
GID1 – GID1a, GID1b and GID1c	Gibberellin insensitive 1/1a/1b/1c
GL1/3	GLABARA1/3
GPA1	G protein α subunit 1
GSA	Glutamate semialdehyde
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
GSTF10	Glutathione S-transferase 10
GTG1/2	G protein coupled receptor Type G protein 1/2
HDA16/19	Histone deacetylase 6/16/19
HDAs	Histone deacetylases
HR	Hypersensitive response
Hrp	Hypersensitive response and pathogenicity cluster
HRP	Horseradish peroxidase
HSC70-1/5	Heat Shock Cognate protein 70-1/5
IP5	Inositol pentakisphosphate
IPL	Isochorismate pyruvate lyase
JA	Jasmonic acid
JA-Ile	(+)-7-*iso*-jasmonoyl-isoleucine
JAZ1-12	Jasmonic Acid ZIM domain repressor protein 1-12
JAZ1ΔC	Jasmonic Acid ZIM domain repressor protein 1 without a C terminal
JAZΔJas	Jasmonic Acid ZIM domain repressor protein without a Jas domain

JID	Jasmonic Acid ZIM domain repressor protein interaction domain
KAT1	Potassium channel in *Arabidopsis thaliana* 1
KS	*Ent*-kaurene synthase
LD-PCR	Long distance polymerase chain reaction
LHT1/2	Lysine-histidine transporter
LiAc	Lithium acetate
LOX	Lipoxygenase
LRR	Leucine rich repeat
MAPK	Mitogen-activaed protein kinase
MFP	Multifunctional protein
NB-LRR	Nucleotide binding leucine rich repeat
NCED	9-*cis*-epoxycarotenoid dioxygenase
NDR1	Non-race specific disease resistance 1
NEB	New England Bioscience
NINJA	Novel Interactor of JAZ
NPR1/NPR2/NPR3	Non-expressor of pathogenesis related genes 1
OAT	Ornithine-delta-aminotransferase
OPC6	3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-hexanoic acid
OPC8	3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoic acid
OPDA	(9S, 13S)-12-oxo-phytodienoic acid
OPR3	Oxophytodienoate reductase
ORA59	Octadecanoid responsive *Arabidopsis* 59
P5C	Pyrroline-5-carboyxlate
P5CDH	Pyrroline-5-carboyxlate dehydrogenase
P5CR	Pyrroline-5-carboyxlate reductase
P5CS	Pyrroline-5-carboxylate synthetase
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline with 0.05% Tween20
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDH	Proline dehydrogenase
PEG	Polyethylene glycol
PIF3/4	Phytochrome interacting factor3/4
PP2C	Type 2C protein phosphatase
PR	Pathogenesis related
PR-1/2/5	Pathogenesis related gene 1/2/5
ProDH1/2	Proline dehydrogenase 1/2
PRR	Pattern recognition receptors
PTI	Pathogen associated molecular pattern triggered immunity
PtsD	*Pseudomonas syringae* pv. *tomato* DC3000
PtsD *cor / cor*	PtsD unable to biosynthesize coronatine
PVDF	Polyvinylidene fluoride
PXA1	ATP binding casette transporter
PYL	Pyrabactine resistance like 4/5/6
PYR	Pyrabactine resistance
R protein	Resistance protein
RAN1	Responsive to antagonist 1
RCAR	Regulatory component of Absisic acid receptor
RGA	Repressor of Giberellin
RGL1/RGL2/RGL3	Repressor of Giberellin like 1/2/3
RIPK	RIN4 induced protein kinase
ROS	Reactive oxygen species
S-AdoMet	S-adenosyl-methionine
S. cerevisae	*Saccharomyces cerevisae*
SA	Salicylic acid
SAR	Systemic accquired resistance
SCF^{COI1}	SKP2/ASK1/CULLIN F-box associated with COI1
$SCF^{SLY1/SNZ}$	SKP2/ASK1/CULLIN F-box associated with SLY1/SNZ

SD/-Leu	YPDA media that does not contain leucine
SD/-Trp	YPDA media that does not contain tryptophan
SDS	Sodium dodecyl sulfate
SDS/PAGE	Sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SLAC1	Slow anion channel associated 1
SnRK2	SNF1 related protein kinase
SPY	SPINDLY
SSA	Succinic semialdehyde
SSADH	Succinic semialdehyde dehydrogenase
T3SS	Type 3 secretory system
TCA	Tricarboxylic acid cycle
TGA	TGACG motif
TIFY	TIF[F/Y]XG motif
TIR	Toll Interleukin-1 receptor
TIR-NB-LRR	Toll Interleukin-1 receptor nucleotide binding leucine rich repeat
TPL	TOPLESS
TPR1/2/3	TOPLESS related 1/2/3
TT8	Transparent testa 8
VSP	Vegetative storage protein
VSP2	Vegetative storage protein 2
X-alpha-Gal	5-Bromo-4-Chloro-3-indolyl α-D-galactopyranoside
Y2HG	Yeast-two-hybrid Gold *Saccharomyces cerevisae*
YPD	Yeast potato dextrose
YPDA	Yeast potato dextrose adenine
ZEP	Zeaxanthin epoxidase

1: Introduction

1.1: Plant Microbe Interactions

As plants have no circulatory system their immune responses must activate local responses and systemic signals. Plants have evolved to recognize certain pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). PRRs are generally transmembrane proteins with an extracellular leucine rich repeat (LRR) domain.[1] An archetypal example of a PAMP is flagellin, an essential component of the bacterial flagellum, in which a highly conserved domain known as flg22 is recognized by flagellin sensing 2 (FLS2) in *Arabidopsis thaliana* (*At* hereafter).[2] FLS2 is a pattern recognition receptor (PRR) protein, containing an LRR domain, that activates a mitogen-activated protein kinase (MAPK) cascade upon recognition of flg22.[3] This MAPK cascade results in a two and a half fold increase in transcription of over 600 genes, amongst which are various defence related genes such as PAD4 and EDS1.[2] Another example is Elongation factor-Tu (EF-Tu) protein found in *Agrobacterium tumefaciens*, which contains a peptide sequence known as elf18 that is recognized by the LRR kinase Elongation Factor-Tu receptor (EFR).[4] EFR activates a different MAPK cascade than FLS2 but it results in the expression of a set of genes that clearly correlates with the genes induced by FLS2.[4] The recognition of PAMPs by plants and the subsequent gene expression is known as PAMP triggered immunity (PTI). During PTI the plant will; express defence related genes, produce reactive oxygen species (ROS), deposit callose and inhibit growth of the pathogen.[5] PTI is essentially the plant "priming" itself against pathogen infection.

In order to successfully colonize the host plant, pathogens suppress PTI by delivering effectors into plant cells using a type three secretion system (see 1.4.1). These effectors (otherwise known as avirulence or Avr proteins) enhance pathogen fitness by manipulating the cellular machinery of the host plant to suppress host defences and produce a more favourable environment for the pathogen, resulting in a phase of plant-pathogen interaction known as Effector Triggered Susceptibility (ETS). Interestingly, the targets of effectors from different pathogens overlap as effectors target "cellular hubs".[6] For example *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*

diverged from a common ancestor 2 million years ago but their effectors still share 18 target proteins in *At*.[6] As an example of an effector *Pseudomonas syringae* pv. *tomato* DC3000 (PstD) uses a molecular mimic of the hormone conjugate jasmonic acid – isoleucine (JA-Ile), the phytotoxin coronatine, to suppress plant defences.[7] In the case of PstD, coronatine deficient mutants are unable to grow to wild type levels, induce systemic induced susceptibility or to produce typical symptoms in *At*.[8,9]

Effectors are delivered into the plant cell by pathogens, where they are directly or indirectly perceived by plant resistance (R) proteins. R proteins are Nucleotide Binding – Leucine Rich Repeat (NB-LRR) domain containing proteins with a centrally located NB domain and a C terminal LRR domain.[1] The N terminal can contain a Toll-Interleukin-1 Receptor (TIR) or a Coiled-Coil (CC) domain; these determine the R proteins' interactions downstream.[1] TIR-NB-LRR proteins interact with Enhanced Disease Susceptibility 1 (EDS1) while CC-NB-LRR proteins require Non-race specific Disease Resistance 1 (NDR1) to continue the signalling cascade.[10] Effectors are directly or indirectly recognized by R proteins resulting in Effector Triggered Immunity (ETI). Indirect recognition involves the R protein sensing the modification of a host protein by pathogen effectors. In *At* an example of indirect recognition involves RIN4 which is targeted by multiple Avr proteins (AvrRpt2, AvrRpm1, AvrB and HopF2) and is guarded by two R proteins (RPS2 and RPM1).[10] AvrRpt2 is a protease that cleaves RIN4 which activates ETI via RPS2. RPM1 is activated by AvrB and AvrRpm1 as RIN4 is phosphorylated in the presence of these two Avr proteins by RIN4 Induced Protein Kinase (RIPK).8,[1] RIPK does interact with AvrB, which suggests that AvrB interaction with RIN4 and RIPK induces the RIPK mediated phosphorylation of RIN4 (see Fig. 1).[1] Direct recognition of effectors involves a direct interaction between R and Avr proteins. For example the *At* resistance protein RPP1 directly associates with ATR1, an oomycete effector.[11] Whether the interaction is direct or indirect, recognition of Avr proteins results in the activation of ETI and a hypersensitive response (HR) that activates programmed cell death (PCD). As well as an HR other defence responses are activated; expression of pathogenesis related (PR) proteins, ROS production and altered hormone signalling. It should be noted that NB-LRR mediated defences is effective against obligate biotrophs and hemibiotrophic pathogens (such as PtsD) but not necrotrophic pathogens (such as *Botrytis cinerea*).[12] HR is often followed by activation of systemic acquired resistance (SAR) which is a systemic priming of the plants resistance against a

broad variety of plant pathogens.[13] SAR is characterised by an increase in salicylic acid (SA), the expression of *PR* genes (e.g. *PR1*, *PR2*, *PR5*) and enhanced resistance to pathogens.[14,15]

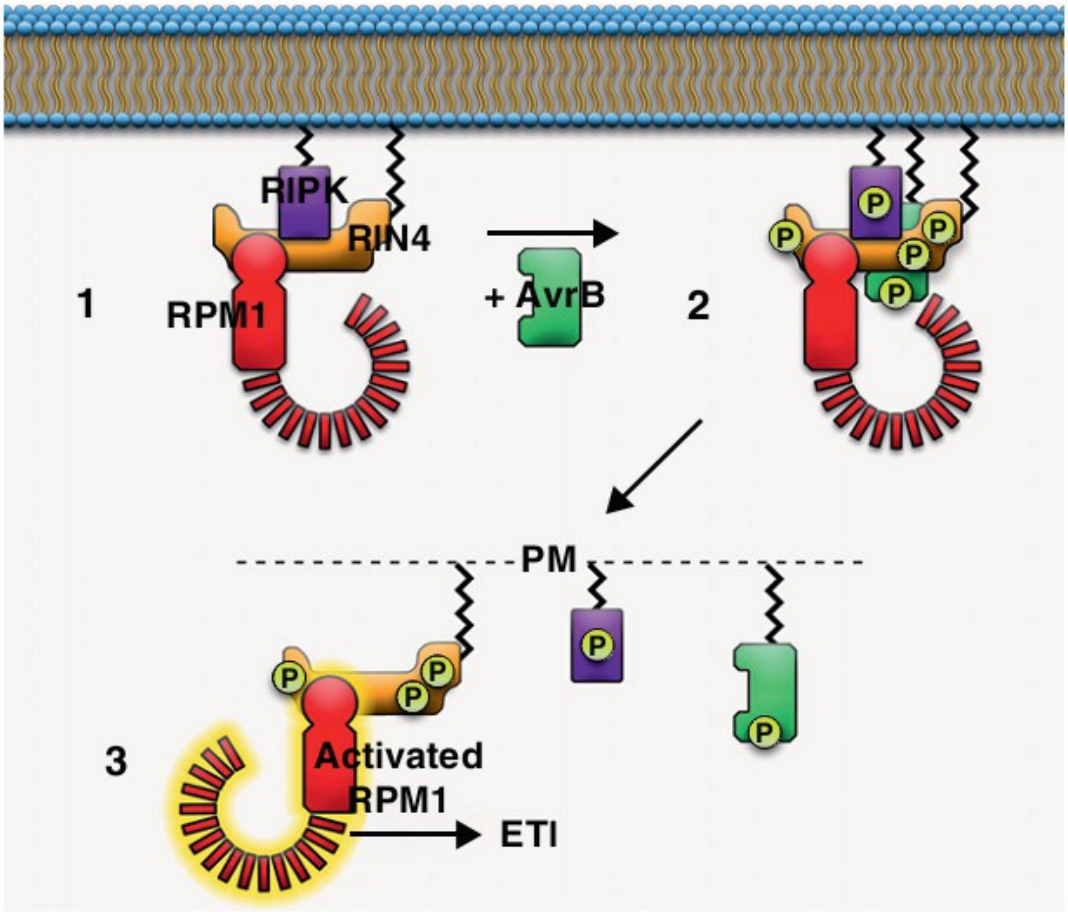


Fig.1 Activation of ETI by AvrB via RPM1. 1. At steady state RIPK, RIN4 and RPM1 form a complex 2. AvrB has been secreted into the cell via PstDs type 3 secretion system and associates with RIN4 and RIPK which induces RIPK mediated phosphorylation of AvrB and RIN4. The phosphorylation of RIN4 causes the dissociation of AvrB and RIPK from the complex and RPM1 to perceive the phosphorylation of RIN4 triggered a signalling cascade.[10]

1.2: Phytohormones and Plant Defence

Plant hormones are key signalling molecules in plants. They are essential for integrating different signals and play important roles in development, growth, defence and cell death. There are many different plant hormones but we will focus on abscisic acid (ABA), ethylene (Et), gibberellin (GA), jasmonic acid (JA) and salicylic acid (SA). The cross talk between these hormones is carefully orchestrated in plants so pathogens manipulate these pathways to suppress plant defences and facilitate their own growth.

1.2.1: Jasmonic Acid

Jasmonic acid (JA) is a oxylipin signalling molecule involved in responses to environmental and developmental signals. JA is involved in many processes including; trichrome and stamen development, fertility, root growth, sex determination, responses to ultraviolet radiation, senescence and activation of plant defence responses[16–20].

1.2.1.1: Jasmonic Acid biosynthesis

Biosynthesis of JA in *At* begins in the chloroplasts with α-linoleic acid being released from glycerolipids in the chloroplast membrane by defective in anther dehiscence 1 (DAD1). α-linoleic acid is then oxygenated by lipoxygenase (LOX) to form 13 (S)-hydroperoxylinolenic acid.[21] In *At* leaves it has been suggested that DAD1 only acts late in wound response stimulated generation of JA and that other phospholipases are responsible for pathogen stimulated generation of JA.[21] 13 (S)-hydroperoxylinolenic acid is used as a substrate by allene oxide synthase (AOS) to generate 12,13 (S)-epoxylinolenic acid, which is cyclized by allene oxide cyclase (AOC). AOC produces (9S, 13S)-12-oxo-phytodienoic acid (OPDA), which is almost exclusively the stereoisomer detected in wounded plants, and dinor-OPDA from 16:3α-linoleic acid.[22] OPDA and dinor-OPDA are transported to the peroxisome, in a manner that is partially dependent on an ATP binding cassette transporter PXA1, where they are reduced to cyclopentanones by oxophytodienoate reductase (OPR3).[22,23] The respective resultant compounds 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoic acid (OPC8) and 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-hexanoic acid (OPC6) undergo three rounds of β-oxidation, catalysed by three core enzymes; acyl-CoA oxidase (ACX), multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase, to produce jasmonoyl-CoA.[17,19,22,23] According to Schaller *et al.* 2009 there likely exists an acyl-thioesterase

that is responsible for the hydrolysis of jasmonoyl-CoA to jasmonic acid. The JAR1 enzyme, located in the cytoplasm, is responsible for the conjugation of isoleucine to jasmonic acid resulting in the production of (+)-7-*iso*-jasmonoyl-isoleucine (JA-Ile) which is the most biologically active form of JA.[24,25] However jasmonic acid is a precursor to many other biologically active derivatives; methyl-jasmonate, cis-jasmone, tuberonic acid and cucurbic acid.[22]

1.2.1.2: Jasmonate Zim Domain Repressors

Jasmonate Zim Domain Repressor (JAZ) proteins are members of the TIFY family of proteins, named after the highly conserved TIFY (TIF[F/Y]XG) motif which lies in the Zinc-Finger Expressed In Inflorescence Meristem (ZIM) domain.[18,26] The ZIM domain of the JAZ proteins does not contain a GATA-type Zinc finger domain, so the JAZ proteins are part of group II of the TIFY family.[26] The JAZ protein family in *At* consists of twelve JAZ proteins (JAZ1-JAZ12), which are predicted to produce over 20 splice variants.[27] Their expression is rapidly induced following de-repression of the MYC2 transcription factor which occurs during jasmonate signalling (see 1.2.1.5).[28] The JAZ family has high sequence variability but posses three conserved domains; the N-terminal (NT), ZIM and Jas domain.[16,29] The NT domain is largely uncharacterised although it has been shown to be important for interactions between JAZ1 and Repressor of GA1-3 (RGA), so this domain is involved with phytohormone cross talk.[16,30]

The ZIM domain consists of 30 amino acids in the centre of the JAZ proteins that contains a highly conserved TIFY motif.[26] The TIFY motif is necessary and sufficient for JAZ-JAZ hetero/homodimerization, which can take place independent of the presence of JA-Ile.[31–33] This dimerization may be important for JAZ localization in the nucleus as nuclear bodies.[34] The ZIM domain is also responsible for the interaction with Novel Interactor of JAZ (NINJA). *Pauwels et al,* 2010 demonstrated that a 39 residue fragment containing the TIFY motif was sufficient for interaction with the C domain of NINJA that mediates NINJA-JAZ interaction.[31]

The Jas motif located towards the C-terminus, is necessary and sufficient for the interaction with COI1 (see 1.2.1.5) and the transcription factor MYC2 (see Fig 2).[18] Inside the Jas motif is a conserved sequence known as the JAZ degron (comprised of Glu200-Val220) which is necessary and sufficient for JAZ-COI1 interaction and

contains the highly conserved LPIARR sequence.[35] The sequence is largely conserved amongst the JAZ proteins (JAZ1,2,3,10,11,12 have fully conserved sequences) but JAZ7 and JAZ8 contain a PKASM sequence instead of LPIARR and these two JAZs have not been shown to interact with COI1.[36] However it has been demonstrated that JAZ8-COI1 interaction can be restored by restoring the LPIARR sequence.[36] JAZ-MYC interactions are not affected by the LPIARR sequence so the sequence determining JAZ-MYC interactions is different.[37] It has been thought that the Jas motif might play a part in the nuclear localization of JAZs due to the presence of a nuclear localization signal in the motif, although JAZ variants lacking the motif do still localize to the nucleus.[34] *Chung et al*, 2009 suggest that the high p*I* of JAZ proteins may assist their localization to the nucleus.[18]

1.2.1.3: NINJA, Topless and other co-repressors

NINJA is a nuclear localized protein related to the ABI-Five Binding protein (AFP) family, whose expression is induced by methyl jasmonate.[31] The C domain of NINJA interacts with the TIFY motif of JAZ proteins, apart from JAZ 7 and JAZ8. Although JAZ7 and JAZ8 contain a TIFY motif, the motif contains an asparagine residue at the fifth position of the motif (TIFYNG). *Wagner and Browse,* 2012 suggest that this residue could be the reason for the lack of a JAZ7/8-NINJA interaction in a Yeast Two Hybrid assay however JAZ10 contains the same TIFY sequence (TIFYNG) so this residue could not be hindering the interaction with NINJA.[16,29] NINJA has been shown to co-purify with the Groucho/Tup1 co-repressor TOPLESS (TPL), TOPLESS RELATED PROTEIN 2 and 3 (TPR2 and TPR3).[31] TPL interacts with an ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif of NINJA, in this way NINJA is able to recruit TPL co-repressors to the JAZ-Transcription factor complex.[31] There are actually several JAZ proteins that contain EAR motifs (JAZ5,6,7,8) and it has been shown that JAZ5, JAZ6 and JAZ8 are able to recruit and directly interact with TPL without NINJA.[36,38,39] EAR motifs are also implicated in the recruitment of Histone Deacetylases (HDAs).[30,36,39–41] TPL and TPR1 have been shown to associate with Histone Deacetylase 19 (HDA19) *in vivo* and HDA19 mutants have weakened pathogen resistance.[42] Interestingly HDA6 interacts with JAZ1, JAZ3 and JAZ9 to decrease the transcriptional ability of ethylene responsive genes, implicating HDAs in phytohormone cross talk.[16,43] HDAs are recruited via EAR motifs and alter chromatin structure, an HDA inhibitor trichostatin A was able to induce ERF1

expression (see 1.2.2) and root hair formation (which is mediated by JA). This indicates that histone deacetylation is a major part of the JA regulatory system.[41,43] This information suggests that JAZs form a regulatory complex where JA responsive transcription factors are bound to JAZs, while JAZs recruit HDAs via NINJA and TPL.

1.2.1.4: Jasmonic Acid Regulated Transcription Factors

The first JA responsive transcription factor to be identified was the nuclear localized basic-helix-loop-helix (bHLH) protein MYC2 which controls JA regulated responses to necrotrophic pathogens and wounding (both biotic and mechanical).[44] *Fernandez-Calvo et al,* 2011 discovered two homologs of MYC2 named MYC3 and MYC4; similar to MYC2 in their interactions with JAZ proteins which occur between the N-terminal JAZ Interacting Domain (JID) of the MYCs and the JAZs C-terminal Jas domain (see also Fig. 2).[20,45] MYC3 and MYC4 interact with all JAZs in a yeast two hybrid screening except for JAZ4 which only MYC4 weakly interacts with. However unlike MYC2; MYC3 and MYC4 did interact with JAZ7.[45] It should be noted that MYC2 interacts with all 12 JAZs.[46] The MYC transcription factors display the highest binding affinity to promoter regions containing the canonical G-Box sequence (CACGTG) and display the same preference for 5' and 3' nucleotides.[45] The MYCs form hetero and homodimers *in vivo.*[45] MYC2 strongly self-induces expression after increases in JA but MYC3 and MYC4 are not induced by JA.[45] The MYCs act additively on JA induced defence genes *PDF1.2* and *VSP2*, a triple knockout (*At-myc2-myc3-myc4)* mutant was almost as impaired in the expression of *PDF1.2* and *VSP2* as *At-coi1-1* which is completely JA insensitive.[45] The importance of the MYCs is further highlighted as a *At-myc2-myc3-myc4* mutant was as impaired as a *At-coi1-1* mutant in resistance to PstD and *Spodoptera littoralis*.[45] This shows that the MYC transcription factors function both in resistance to hemibiotrophic pathogens and insect herbivory. The triple MYC mutant is not completely compromised in JA responses showing there are more transcription factors that act redundantly with the MYCs, at least for developmental purposes such as root inhibition and fertility.[45]

JAZ proteins also interact with the WD-repeat/bHLH/MYB complex which regulates JA induced anthocyanin accumulation and trichrome initiation.[16,46] Anthocyanins are important antimicrobial agents that act against insect herbivory and pathogen infection.[46,47] Trichromes are hair-like structures, produced on the aerial epidermis of

leaves and stems. They function in defence against herbivores inhibiting the movement of herbivores and releasing toxic chemicals.[48] bHLH family proteins such as GLABARA3 (GL3), ENHANCER OF GLABARA3 (EGL3) and TRANSPARENT TESTA8 (TT8) form a complex with a R2R3 MYB protein (GL1, MYB75, MYB113, MYB114, MYB23) and TTG1 (a WD-repeat protein). JAZ proteins (JAZ1,2,5,6,8,9,10,11) repress the WD-repeat/bHLH/MYB complex through interaction with the C-terminal of bHLH proteins and R2R3 MYB proteins likely via the Jas domain.[46] A diagram of these interactions is displayed below in Fig. 2.

1.2.1.5: Jasmonic Acid Signalling

During normal conditions Jasmonate Zim Domain (JAZ) proteins along with the co-repressors NINJA, TPL and TPR inhibit the transcription of JA responsive genes by binding to JA responsive transcription factors like MYC2 (see Fig 2. and 1.2.1.4). When levels of JA-Ile rise, the hormone is perceived by a COI1-JAZ co-receptor, the JAZ-COI1 interaction is promoted by JA-Ile (see Fig 3).[37] COI1 is part of a SKP2/ASK1, CULLIN, F-box protein complex known as SCF^{COI1}, an E3 ubiquitin ligase.[16,17] The presence of JA-Ile cause a successful interaction between JAZ proteins and SCF^{COI1} leading to the ubiquitination of JAZ proteins which causes them to be degraded by the 26S proteasome.[28] The newly derepressed JA responsive transcription factors are then able to bind the promoters of JA responsive genes, amongst these are genes encoding JAZ proteins which re-establishes repression of JA responsive transcription factors.[28,49] Chini *et al,* 2007 showed that JAZ protein expression increased within 30min of JA treatment.[28] In this way plants use a negative feedback loop to carefully regulate the expression of JA responsive genes.

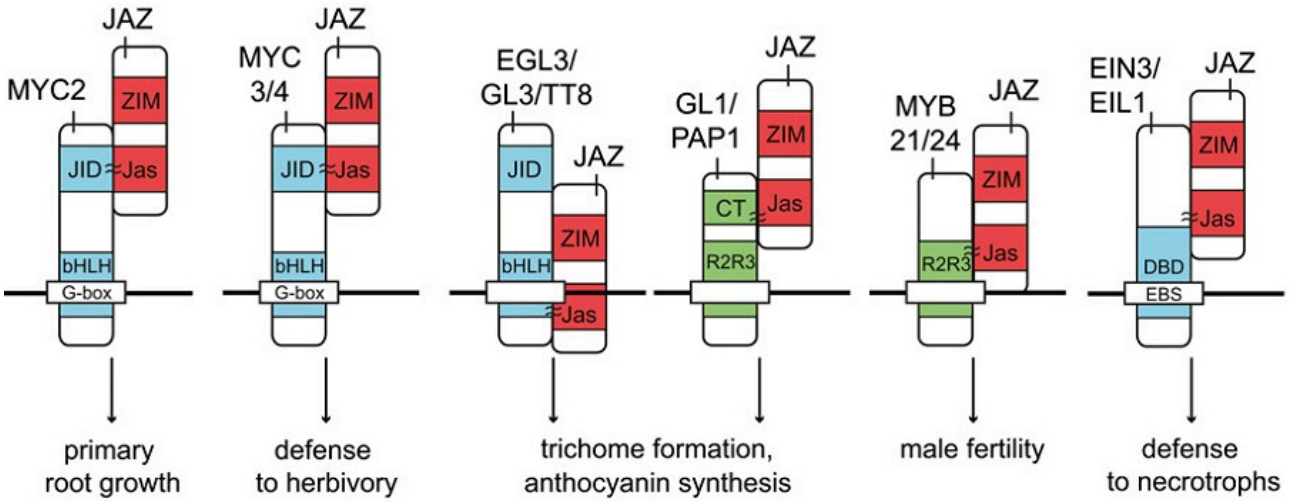


Fig. 2 Interactions between JAZ and various transcription factors. The different processes that the transcription factors mediate are shown below the arrows. The white boxes show the promoter sequences that the transcription factors preferentially bind to. The approximation symbols show the approximate regions where interaction occurs.[20]

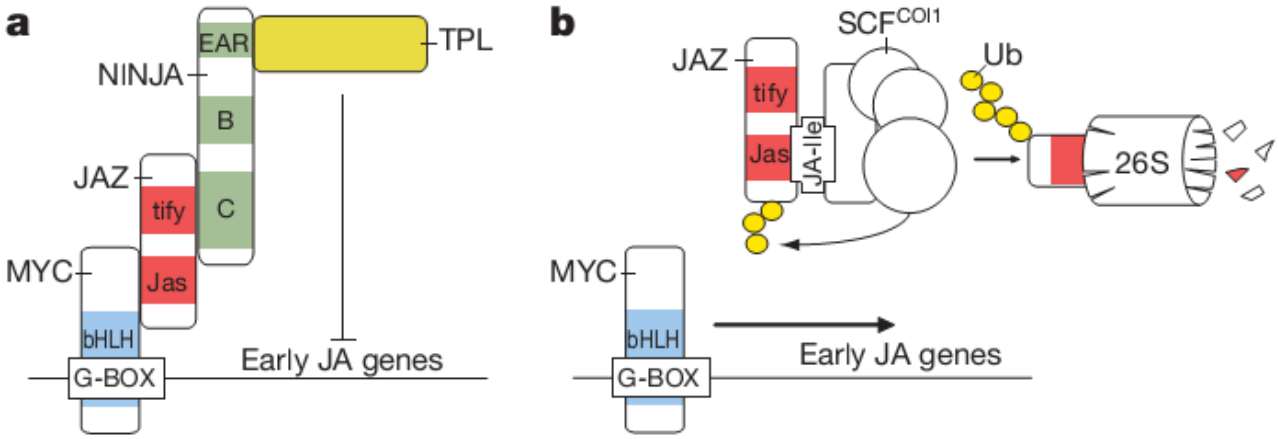


Fig. 3 Model for general function of JAZ in regulating JA response. The repression of MYC by JAZ and co-repressors and de-repression of MYC . a) Shows how the JAZ-NINJA-TPL repressor complex inhibits MYC transcription factors from binding to JA responsive genes. b) Shows the formation of the JA-Ile-JAZ-SCF^{COI1} complex, the subsequent ubiquitination of JAZ proteins and their destruction via the 26S proteasome. The derepressed MYC transcription factor is now able to bind promoter regions of JA responsive genes containing a G-box binding site.[28,31]

COI1 and JAZ form a co-receptor for JA-Ile and the binding of JA-Ile to COI1 is significantly lower without JAZ.[16,35] The JAZ-COI1 interaction is largely dependent on JA-Ile, although JA-Leu (Jasmonic acid conjugated to leucine) has been shown to prompt the interaction.[50] This is because the Jas degron helps form the JA-Ile binding pocket; the LPIARR sequence (see 1.2.1.2) helps to close the JA-Ile binding pocket while the C-terminal of the degron strengthens the JAZ-COI1 interaction.[35] The ARR part of the LPIARR sequence directly interacts with JA-Ile which explains the JA insensitive phenotype of JAZ1 mutants with alanine substitutions in the ARR sequence (ARR → AAR and AAA).[36,37] While JA-Ile is necessary for the COI1-JAZ interaction, inositol pentakisphosphate (IP5) is required for the perception of JA-Ile by the JAZ-COI1 complex. IP5 interacts with three arginine residues in the binding pocket of COI1 and a single arginine residue in the Jas degron (LPIAR**R**) forming a tetragonal bipyramidal interaction between four arginine residues.[25,35,51] IP5 is crucial for high sensitivity perception of JA-Ile by the COI1-JAZ co-receptor, although interestingly COI1 in the presence of inositol tetrakisphosphate was able to bind more coronatine than COI1 in the presence of IP5.[35] Removal of IP5 from the COI1-JAZ complex causes a significant reduction in COI- JAZ1 binding affinity for coronatine (which is a JA-Ile mimic) and alterations in the biosynthesis of IP5 also alters COI1-JAZ interactions.[35,51]

To stop JA-Ile mediated signalling, JA-Ile must be removed from the nucleus to prevent it from stimulating COI1-JAZ interactions. It has been shown that there are two cytochrome P450 enzymes that catabolise JA-Ile; CYP94C1 and CYP94B3.[52,53] CYP94B3 hydroxylates JA-Ile to form 12OH-JA-Ile and CYP94C1 carboxylates 12OH-JA-Ile to form 12COOH-JA-Ile.[52,53] 12OH-JA-Ile does not promote COI1-JAZ interactions to the same level as JA-Ile so the formation of 12-COOH-JA-Ile may be necessary to silence JA signalling. The importance of JA-Ile turnover to JA signalling can be seen in *At-cyp94b3* mutants, these mutants hyperaccumulate JA-Ile and have increased sensitivity to JA-Ile, additionally they have decreased resistance to PtsD.[54] Meanwhile *At-cyp94c1* mutants overexpress JAZs and expression of defence related genes is inhibited, although JA-Ile levels remained similar to wild type levels.[53] Additionally there are also glucosylated and sulphated JA metabolites such as 12-*O*-Glc-JA and 12-HSO_4- JA, although their biological roles (if any) remain unclear. It should be noted that the two CYP94 enzymes cannot metabolize coronatine.[53] Heitz *et al.* 2012 suggest that the catabolism of JA-Ile is necessary as JA-Ile catabolites and

JAZΔJas inhibit the COI1-JAZ interaction with JA-Ile. This would prevent SCF^{COI1}-26S proteasome mediated degradation of JAZs and inhibit further JA-Ile mediated signalling.[53]

1.2.1.6: Alternatively Spliced JAZ proteins

As previously mentioned there are splice variants of JAZ proteins, some of which lack the Jas domain or part of the Jas domain. These variants are known as JAZΔJas. Overexpression of splice variants confers reduced sensitivity to JA, impaired transcriptional activation of JA responsive genes, male sterility and enhance resistance to PstD.[25,28,29] Given that the Jas domain is necessary and sufficient for the binding of JAZ proteins to COI1, JAZ proteins lacking a Jas motif (e.g. JAZ10.4, JAZ7, JAZ8) do not interact with COI1 and are therefore stable in the presence of JA-Ile.[25,28,29,36] The JAZΔJas arise because in nine of the 12 JAZ proteins the 27 amino acid Jas motif is split by an intron (Jas intron) into 20 N-terminal and seven C-terminal amino acid segments, alternative splicing can lead to this intron being retained which generates a premature stop codon.[29] The nine JAZs (JAZ2,3,4,5,6,9,10,11,12) that contain the Jas intron have been shown to produce mRNA transcripts that lack the entire Jas motif.[27] JAZ10.4 which lacks the entire Jas motif, was shown to be highly resistant to JA induced degradation and in an over expression line 35S-JAZ10.4 plants were highly insensitive to JA-induced root growth inhibition.[27,29]

The function of alternatively spliced JAZ proteins has yet to be elucidated but *Chung and Howe,* 2009 suggest that their function is to regulate the response to JA in stimulated cells for example to wounding or insect herbivory, whereas full length JAZ proteins regulate responses to JA in unstimulated cells. They also suggest that JAZ10.4 is an endogenous repressor of JA mediated responses, as *At* JAZ10 RNA interference silenced plants display a hypersensitivity to JA that does not occur in other *At* JAZ RNA interference silenced plants.[29] There is also the suggestion that JAZΔJas protect full length JAZs from SCF^{COI1} mediated destruction by dimerization with the full length proteins, dampening the response to JA by interfering with JAZ degradation.[16] Another possibility is that alternatively spliced JAZs (JAZs with sequence variability in the Jas region) and JAZ7/JAZ8 give the plant the ability to respond to varying levels of JA-Ile, as various levels of JA-Ile promote different JAZs binding to COI1. For example JAZ6 lacks conserved N-terminal residues in the LPIARR region of the Jas degron, which reduces the binding capacity to COI1 as compared to JAZ2 or JAZ12; therefore higher

levels of JA-Ile are required to promote JAZ6-COI1 binding as compared to JAZ2/JAZ12-COI1.[36] This serves as an example of how JAZs can respond to varying levels of JA-Ile as their binding to COI1 requires varying threshold levels of JA-Ile. JAZ7 and JAZ8 expression is highly upregulated in response to wounding and infection with PstD whose phytotoxin coronatine is approximately 1000 fold more effective than JA-Ile at promoting COI1-JAZ1 interaction.[55] Given the resistance of JAZ7 and JAZ8 to JA induced destruction this suggests that JAZs deficient in COI1 interaction (e.g. JAZ7, JAZ8, JAZ10.4) may act as part of a negative feedback loop to prevent runaway JA responses that would lower plant fitness.[7,56]

1.2.2: Ethylene

Ethylene is a gaseous plant hormone involved in seed germination, root hair development, flower senescence, abscission and fruit ripening. Ethylene is produced by the metabolism of methionine to S-adenosyl-methionine (S-AdoMet), almost 80% of cellular methionine is converted to S-AdoMet by S-adenosyl-methionine synthetase.[7] S-AdoMet is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by S-adenosyl-L-methionine methylthioadenosine-lyase (ACC synthase), which is then oxidized by ACC oxidase to ethylene, carbon dioxide and cyanide.[43,57]

Ethylene is perceived by five endoplasmic reticulum localized receptors; ethylene response 1 (ETR1), ethylene response 2 (ETR2), ethylene response sensor 1 (ERS1), ethylene response sensor 2 (ERS2) and ethylene insensitive 4 (EIN4) that are homologous to bacterial histidine kinases.[57] The receptors are negative regulators of the ethylene signalling pathway and interact with a Raf-like kinase; constitutive triple response 1 (CTR1). CTR1 represses ethylene insensitive 2 (EIN2), an essential positive regulator of ethylene responses downstream which leads to the inactivation the transcription factors; Ethylene insensitive 3 (EIN3) and EIN3-like Protein 1 (EIL1).[57,58] In the absence of ethylene EIN2 is targeted by EIN2 targeting protein 1 (ETP1) and EIN2 targeting protein 2 (ETP2), while EIN3/EIL1 are targeted by EIN3 binding F-box proteins1 and 2 (EBF1/2) for ubiquitination/degradation.[43,57,58]

Ethylene and a copper containing co-factor, supplied by a golgi-localized Responsive to Antagonist 1 (RAN1) copper transporter, bind to the ethylene receptors (ETR1, ETR2, ERS1, EIN4) inactivating CTR1 and through the downregulation of ETP1 and ETP2 allow the accumulation of EIN2.[59] EIN2 regulates a transcriptional cascade in the

nucleus that is initiated by EIN3 and EIL1, which results in the activation of the transcription factors Ethylene response factor 1 (ERF1) and Ethylene response DNA binding factors 1-4 (EBF1-4) and an increase in the expression of ethylene responsive genes (See Fig. 4).[43,57–59]

Et and JA are vital for plant development and defence against the necrotroph *Botrytis cinerea* as they act synergistically to suppress SA responses, as well as inducing various pathogenesis related genes.[60] The two hormones induce expression of similar sets of genes, a microarray assay showed that 50% of the genes induced by Et treatment were also induced by treatment with MeJA.[43,61] Overexpression of Ethylene response factors 5 and 6 (ERF5/6) in *At* resulted in expression of defence genes and JA/Et responsive genes, which suggests that they are positive regulators of JA mediated defences.[62] Constitutive expression of ERF5/6 in *At* caused significantly increased resistance to *B. cinerea,* whereas *At-erf5/6* mutants were significantly more susceptible and had reduced JA induced gene expression.[63] In the *At-ein3-eil1* double mutant a number of pathogenesis related genes were not expressed, although wounding response genes (whose expression is mediated by MYC transcription factors) were still induced.[43] The same authors also showed that *At-ein3*, *At-eil1* and *At-ein3-eil1* were more susceptible to *B. cinerea* although *At-myc2* was more resistant.[43] *At-ein3-eil1* was partially insensitive to the inhibition of root elongation by JA application but overexpression lines (*At-EIN3ox, At-EIL1ox*) were hypersensitive to JA mediated root growth inhibition.[43] However fertility and JA induced anthocyanin biosynthesis were not affected in *At-ein3-eil1* plants. These results suggest that EIN3 and EIL1 are involved in the positive regulation of some JA genes related to plant defence and root development but not anthocyanin production or fertility.[58] This hormonal crosstalk could be a result of JAZ1, JAZ3 and JAZ9 interacting with EIN3/EIL1 (as they have been shown to do in a yeast two hybrid assay) probably via the Jas domain in the C-terminus of the JAZ proteins (See Fig. 2).[43] The crosstalk is furthered by histone deacetylase 6 (HDA6), the expression of which is upregulated by Et and JA, which interacts with both EIN3, EIL1 and JAZ1, JAZ3 and JAZ9.[43] The HDA6-JAZ1 interaction still occurs with JAZ1ΔC, therefore the interaction is not mediated by the Jas domain and interactions between JAZ-EIN3/EIL1 and JAZ-HDA6 occur between distinct domains.[43,64] HDA6 is recruited by JAZs to deacetylate histones in order to obstruct EIN3 and EIL1 chromatin binding thereby preventing further expression of Et-responsive genes.[43] Use of an HDA

inhibitor is sufficient to strongly induce ERF1 expression and JA treatment causes a significant increase in the level of histone acetylation in the ERF1 promoter region.[43] These data support a model where JAZ proteins recruit HDA6, probably via the co-repressors NINJA and TPL, as a co-repressor to inhibit the activity of EIN3/EIL1. The JAZs bind to EIN3/EIL1 and HDA6 deacetylates chromatin to repress the transcriptional ability of EIN3/EIL1. In the presence of JA and Et; Et stabilizes EIN3 and EIL1 by proteasomal degradation of EBF1 and EBF2, JA causes degradation of JAZs via SCF^{COI1} 26S proteasome pathway which allows expression of JA/Et responsive genes.[43] A diagram of the hormonal crosstalk is shown below in Fig.5.

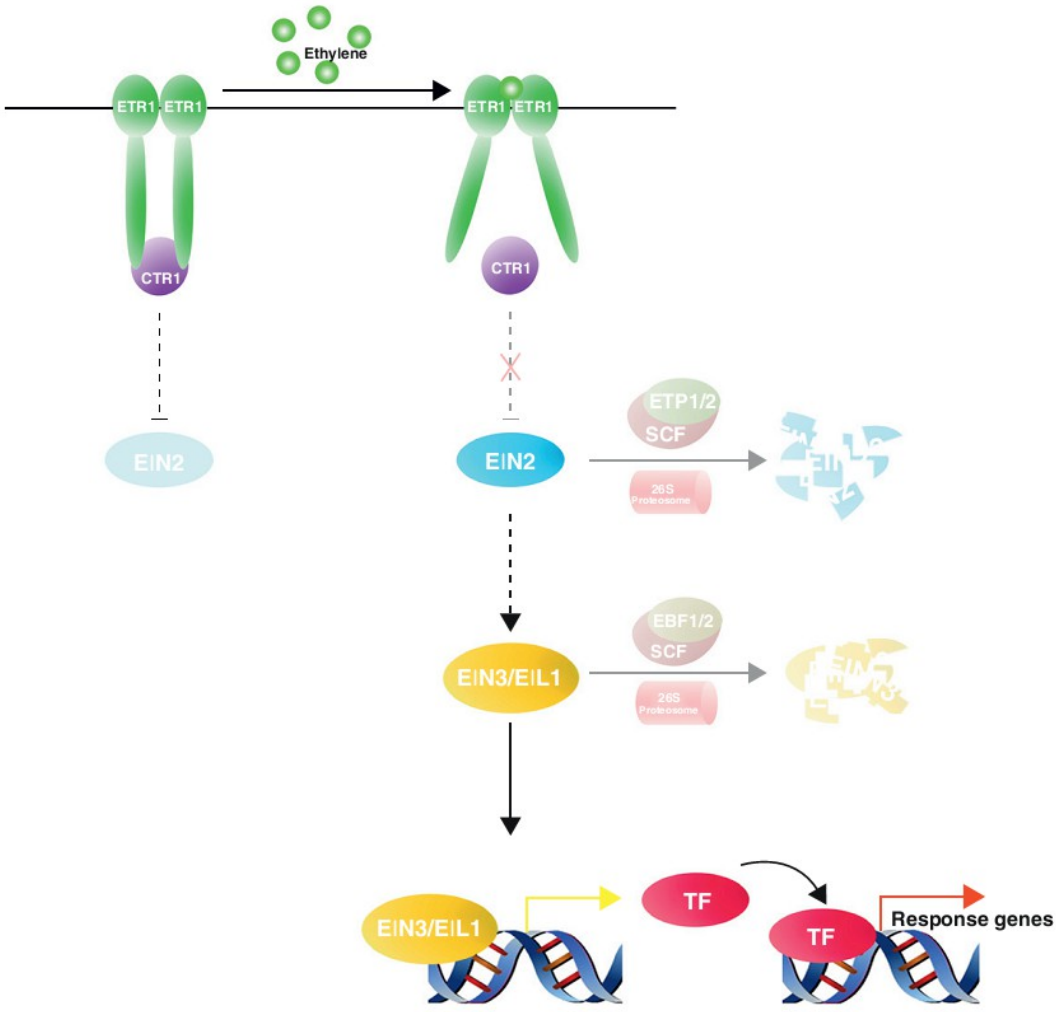


Fig. 4 Model of the ethylene signalling pathway. ETR1 interacts with CTR1 to inhibit EIN2, in the absence of ethylene. Upon ethylene binding to ETR1, ethylene inactivates CTR1 mediated repression of EIN2 leading to EIN2 accumulation. EIN2 mediated transcription of EIN3/EIL1 increases and downstream ethylene responses are activated. In the absence of ethylene EIN2, EIN3 and EIL1 are degraded by the 26S proteasome. Solid lines are direct interactions and dotted lines are incomplete interactions.[58]

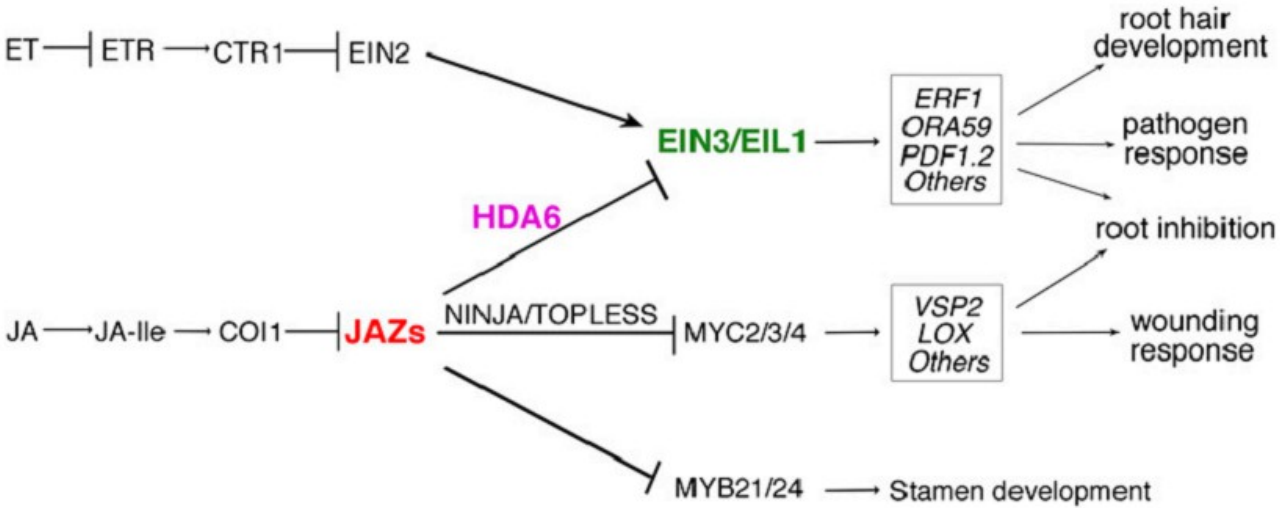


Fig.5 Crosstalk between JA and Et signalling pathways. Et represses the action of ETR on CTR1, derepressing EIN2 upregulates the expression of EIN3 and EIL1. EIN3/EIL1 are then free to upregulate the transcription of Et responsive genes. JA is metabolised to its' bioactive form JA-Ile which causes SCF^{COI1} to mediate the destruction of JAZ proteins, derepressing MYC2/3/4 and MYB21/24. Without the presence of JA, JAZs recruit HDA6 via the co-repressors NINJA and TPL. JAZs bind to EIN3/EIL1 and HDA6 deacetylates histones to repress the transcriptional activity of EIN3/EIL1. Therefore in the presence of both hormones, EIN3/EIL1 are stabilized by Et and derepressed by JA.[28,65]

1.2.3: Gibberellin

Gibberellins (GA) are diterpene hormones that are required for plant development.[43] The major growth process that GAs are involved in include elongation or expansion of; stem, hypocotyl, roots, stamens and pistils cells as well as promoting cell division.[66] GAs are also involved in flower and seed development as well as germination.[16] It has also been shown that GA promotes JA expression while attenuating salicylic acid signalling which enhances *At* resistance to necrotrophs.[16,67–69] GAs are produced in the plastid through the metabolism of *trans*-geranylgeranyldiphosphate (GGPP).[70] GGPP is metabolised to *ent*-kaurene via *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS).[67,71] *ent*-kaurene is oxidized by cytochrome P450 monoxygenases (P450s), P450s are located on the plastid envelope and endoplasmic reticulum (ER), to form GA_{12}.[71] GA_{12} is converted to the bioactive GA_4 by 2-oxoglutarate-dependent dioxygenases (2ODDs), GA 20-oxidase converts GA_{12} to GA_9 which is then metabolised into GA_4 by GA 3-oxidase. In *At,* GA_4 is the most bioactive

GA but GA_1, which contains an OH group at C13 rather than a sole hydrogen, is also bioactive.[71] It should be noted that the GAs are named after the order in which they were discovered and that the name does not relate to its structural derivation nor the organism in which the GA was found.[67,71]

GA is perceived by three GA insensitive dwarf 1 (GID1) receptors; GID1a, GID1b and GID1c.[72] GA and the GID1 receptor form a complex GA-GID1 that is able to bind DELLA proteins. In *At* there are five DELLA proteins; GA insensitive 1 (GAI1), Repressor of GAI1-3 (RGA), RGA-like 1 (RGL1), RGA-like 2 (RGL2) and RGA-like 3 (RGL3).[58,69,73] They belong to the GRAS family of transcriptional regulators and contain a DELLA domain and a conserved GRAS domain.[58,73] The role of DELLA proteins is to bind to GA-responsive transcription factors such as phytochrome interacting factors (PIFs) and Alcatraz (ALC), in order to suppress their DNA binding and subsequent expression of GA responsive genes.[73,74] For example phytochrome interaction factor 3 and 4 (PIF3/PIF4) interact with DELLA proteins rendering the two transcription factors unable to bind their target gene promoters.[58,74,75] PIF3 and PIF4 have been shown to function in light regulated growth and development.[76] In the presence of GA, the GA-GID1-DELLA complex is formed which potentiates the interaction between DELLA proteins and $SCF^{SLY1/SNZ}$ which causes the subsequent ubiquitination and degradation of the DELLA repressor protein.[74,75] The degradation of DELLAs via the $SCF^{SLY1/SNZ}$ - 26S proteasome pathway derepresses transcription factors (such as PIF3) and causes the transcription of GA-responsive genes.[58,69,73] There is evidence that DELLA activity is further modulated by; O-GlcNacylation by SPINDLY (SPY), phosphorylation by a casein kinase and the formation of GA-GID1-DELLA complex that are not degraded by $SCF^{SLY1/SNZ}$.[58]

GA and JA crosstalk is proposed to have an important role in controlling periods of defence and growth. DELLA proteins are able to interact with JAZs and thereby function as competitive inhibitors for JAZ-Transcription Factor complexes such as JAZ-MYC2. For example JAZ1, JAZ3 and JAZ9 have been shown to interact directly with some RGA, via the JAZ NT and Jas domains and DELLA LZ1 and DELLA domains.[77] The MYC2-JAZ1 interaction is weakened in a dose dependent manner by RGA, conversely RGA-JAZ1 interaction is weakened by MYC2 in a dose dependent manner, additionally the MYC2-JAZ3 interaction has also been shown to be antagonized by RGA in a dose dependent manner.[70] The interaction between JAZ1 and DELLA proteins

prevents JAZ1 mediated repression of MYC2 which allows for the expression of MYC2 controlled genes.[78] This crosstalk means that under conditions favourable to growth, GA degrades DELLA proteins which increases JAZ mediated repression of JA responsive transcription factors resulting in further repression of JA signalling. It has been shown that in yeast-two-hybrid assays MYC2 interacts with all five *At* DELLA proteins and as DELLA levels increase and decrease, MYC2 binding to G-boxes increases and decreases respectively.[70] Interestingly JAZ9 is able to disrupt the RGA-PIF3 interaction, which would further encourage GA signalling in the absence of JA signalling.63 Conversely during periods of abiotic or biotic stress JA signalling causes the degradation of JAZ proteins which frees up DELLAs to interact with GA responsive transcription factors repressing GA signalling (see Fig. 6). RGL3 expression is induced by JA in a MYC2 dependent manner, RGL3 binds JAZs releasing more MYC2 and enhancing JA signalling.[79] Interestingly the DELLA proteins positively regulates JA mediated resistance to *B. cinerea* by repressing SA signalling.[80]

1.2.4: Abscisic Acid

Abscisic Acid (ABA) is a terpenoid phytohormone that functions in a number of growth processes including seed dormancy and germination, regulation of stomatal aperture as well as regulation of responses to abiotic stresses such as drought and salinity stress.[81] ABA has been shown to negatively regulate plant resistance to necrotrophic fungi and PtsD.[82,83] PtsD induces expression of ABA synthesis and signalling genes as well as modifying the expression of regulatory components of the ABA pathway causing lowered resistance to PtsD.[81,84] ABA biosynthesis takes place in the plastid although the final steps are localized in the cytoplasm.[84] ABA biosynthesis begins with the breakdown of zeaxanthin into violaxanthin catalysed by zeaxanthin epoxidase (ZEP), violaxanthin's *cis* isomer (how formation of *cis*-violaxanthin occurs is still unknown) is cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to form xanthoxin.[85] Xanthoxin is converted to abscisic aldehyde, in the cytoplasm, via alcohol dehydrogenase (ABA2) which is then oxidised by abscisic aldehyde oxidase 3 (AAO3) to form ABA.[86]

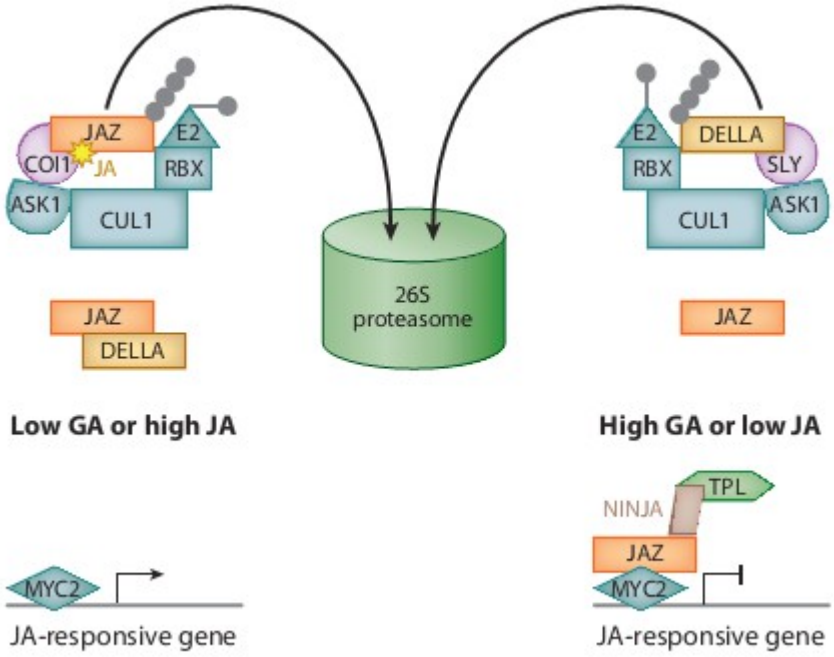


Fig. 6 Model of JA and GA crosstalk. When JA is high JAZs are degraded via SCF^{COI1}. DELLA proteins are stabilized without the presence of GA and bind JAZs preventing JAZs from binding MYC2 thus allowing MYC2 activity to continue. When GA is high DELLA proteins are degraded via SCF^{SLY1} and so JAZs are free to bind MYC2 inhibiting it's activity and preventing expression of JA responsive genes. JAZs are also free to bind MYC2 during periods of low JA.[69]

ABA is proposed to be perceived by three main classes of receptors that function in different subcellular compartments; Pyrabactin resistance/Pyrabactin resistance like/Regulatory component of ABA receptors (PYR/PYL/RCARs), Chelatase H subunit (CHLH) and G protein coupled receptor Type G protein (GTG1 and GTG2).[58,87] PYR/PYL/RCARs bind ABA (see Fig. 7) and form a repressor complex that targets Type 2C protein phosphatases (PP2Cs) and prevents these negative regulators of ABA signalling from repressesing the ABA activating SNF1 related protein kinases (SnRK2s).[58] The ABA-PYR/PYL/RCARs-PP2C interaction causes an accumulation of phosphorylated SnRK2s which leads to phosphorylation of ABA responsive transcription factors like ABA responsive promoter elements (ABREs) binding factors (ABFs), Slow anion channel associated 1 (SLAC1) and of the cation channel protein Potassium channel in *Arabidopsis thaliana* 1 (KAT1).[58,83,87] Phosphorylation of transcription factors causes the transcription of ABA responsive genes. CHLH receptors

are chloroplast transmembrane proteins that bind ABA. ABA stimulates the transport of WRKY40, a transcriptional repressor, from the nucleus to the cytoplasm and enhances CHLH-WRKY40 interaction in the cytoplasm.[58,88] The binding of WRKYs (WRKY40,18 and 60 have been shown to be ABA transcriptional repressors) to CHLH sequesters the WRKY transcription factors removing their repression of ABA responsive transcription factors such as ABA insensitive 5,4 (ABI5/ABI4), ABA binding factor4 (ABF4) and MYB2.[58,88] Lastly GTG1/GTG2 are plasma membrane localized ABA receptors that form a complex with G protein α subunit 1 (GPA1) and guanosine diphosphate (GDP). GPA1 binds and inactivates a phospholipase PLDα1 but ABA binding to the GTG1-GPA1-GDP complex releases PLDα1 and phosphatidic acid promoting ABA responsive gene expression.[88]

ABA plays a complex role in plant defences; exogenous application of ABA represses JA/Et mediated transcription of defence genes *PDF1.2* and *PR4* and increases susceptibility to PstD.[58,87,89] *At* mutants that are defective in their biosynthesis or sensitivity to ABA have increased resistance to PstD, *B. cinerea*, *Fusarium oxysporum* and *Hyaloperonospora arabidopsis*.[81,84] Overexpression of *NCED5*, the ABA biosynthesis gene, results in accumulation of JA and a reduction in SA however the crosstalk between ABA and JA remains unclear. ABA is required for JA biosynthesis and for expression of JA responsive genes upon infection with *Pythium irregulare*.[90] *Lackman* 2011 found that *At-pyl4* and *At-pyl5* mutants are hypersensitive to JA mediated growth inhibition and display reduced anthocyanin accumulation, the authors also found indications that expression of genes related to *PYL4/5/6* can be modulated by JA.[91] Stomatal closure which is positively regulated by ABA has been shown to be repressed by coronatine, a JA-Ile mimic, as well as mutations in ABA and SA biosynthesis genes which signals that there is a crosstalk between ABA and JA/SA pathways.[92,93]

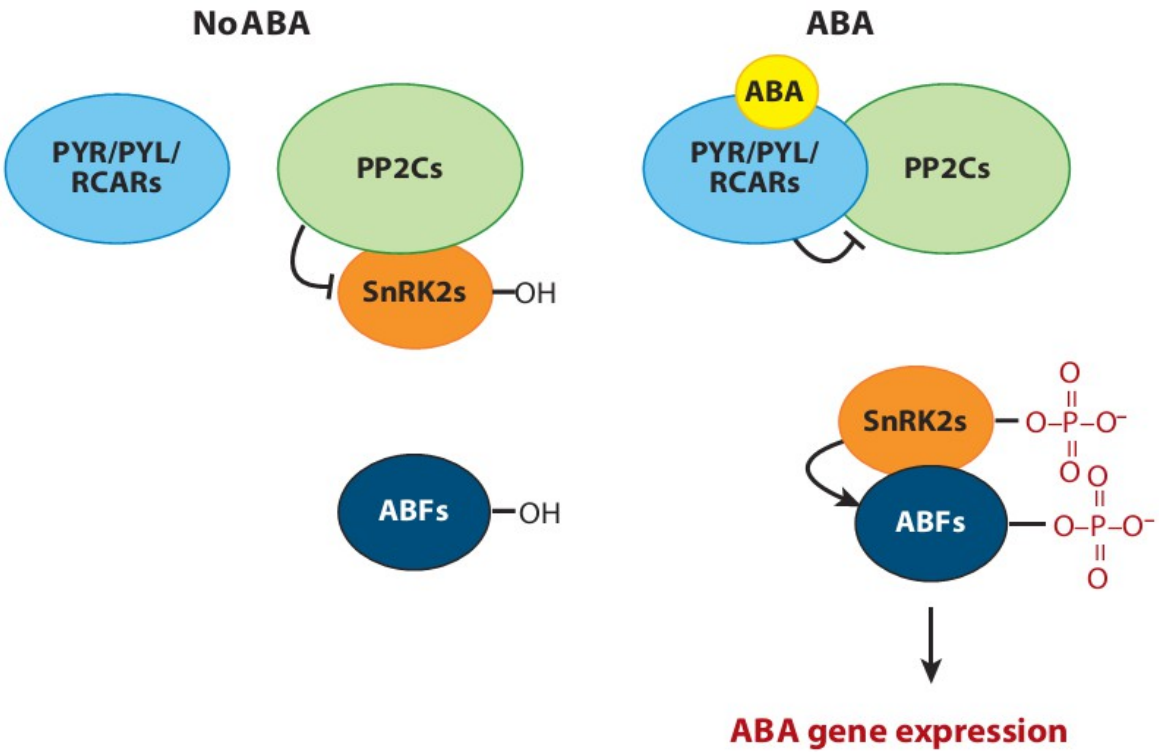


Fig. 7 The ABA regulatory pathway. The figure on the left (No ABA) shows the binding of PP2Cs to SnRK2s in the absence of ABA which suppresses SnRK2 phosphorylation of ABA-responsive element binding factors (ABFs). On the right (ABA) PYR/PYL/RCARs bind PP2Cs in the presence of ABA allowing accumulation of SnRK2s leading to SnRK2 phosphorylation of ABFs and the expression of ABA responsive genes.[94]

1.2.5: Salicylic Acid

2-hydroxy benzoic acid better known as Salicylic acid (SA) is a phenolic compound that regulates plant growth, development, flowering, thermogenesis and importantly plant defence against biotrophic and hemibiotrophic pathogens.[95] As previously mentioned SA is responsible for the induction of SAR, the mechanisms of which will be explained below. There are two proposed biosynthetic routes for SA; the isochorismate pathway and the phenylalanine ammonia-lyase pathway (PAL).[95] The two pathways both take place in the plastid and utilise chorismate in the first step of the pathway but neither pathway is fully defined.[95] The isochorismate pathway begins with the C4 hydroxyl group of chorismate being displaced to C2 forming isochorismic acid which is then reduced to SA by a hypothetical enzyme isochorismate pyruvate lyase (IPL).[95] It should

be noted that no plant gene encoding IPL has been discovered but four chorismate mutase (CM) genes that could perform the function of IPL have been annotated in *At.*

The PAL pathway beings with chorismate being metabolised to phenylalanine via CM, whereupon PAL converts phenylalanine to *trans*-cinnamic acid (TCA). *At* actually contains four PAL encoding genes with differing properties. TCA is potentially converted to benzaldehyde via CoA ligase, whereupon Arabidopsis Aldehyde Oxidase 4 (AAO4) converts benzaldehyde to benzoic acid.[95] It has been suggested that the conversion of benzoic acid to SA occurs via benzoic acid 2-hydroxylase although no gene encoding such an enzyme has been described in plants.[95] The isochorismate pathway appears to be responsible for the majority of SA production in *At* but there is evidence to suggest that another pathway also exists. This is likely to be PAL mediated as in quadruple *At-pal* mutants basal levels of SA were only 25% of of wild type.[95]

Non-expressor of pathogenesis related genes 1 (NPR1) has long been identified as the master regulatory protein in the SA signalling pathway although it was not until recently that NPR3 and NPR4, paralogues of NPR1, were shown to function as receptors in the SA signalling pathway.[96,97] When SA levels are low NPR1 exists in the cytosol as an oligomer bound by intermolecular disulphide bonds. Increasing SA levels cause the cellular redox state to become more reducing which induces the destruction of these bonds via thioredoxins causing NPR1 to become a monomer that localizes to the nucleus.[98,99] NPR1 then binds TGACG (TGA) motif carrying transcription factors (SA increases this binding), along with other transcription factors such as WRKY's, and increases their binding affinity to SA responsive promoters which causes expression of SA responsive defence genes.[81] NPR3 and NPR4 act as Cullin 3 (CUL3) adaptors to bind NPR1, CUL3 is part of an E3 ligase that mediates the degradation of NPR1 via ubiquitination and subsequent destruction by the 26S proteasome.[96] NPR3 and NPR4 have very different binding affinities for NPR1 which are affected by SA. NPR3 binding of NPR1 is promoted by SA whereas NPR4-NPR1 binding is disrupted by SA.[96,97] NPR3 and NPR4 have been shown to bind SA and it has been shown that binding of SA to NPR3 and NPR4 promotes and inhibits binding of NPR1 respectively.[96] This leads to a model (see Fig. 8) where basal levels of SA do not allow NPR3-NPR1 binding, but do allow for NPR4-CUL3 mediated destruction of NPR1, although some NPR1 does remain, which suppress defence responses. The complete destruction of NPR1, such as what occurs in mutants deficient in NPR1 homoeostasis,

results in increased susceptibility to disease.[96] During ETI SA levels increase so NPR3-NPR1 binding is promoted leading to NPR3-CUL3 mediated destruction of NPR1 which in turn leads to PCD and HR. In *At-npr3-npr4* ETI and PCD were inhibited which suggests that NPR1, which accumulates in the mutant, suppresses PCD.[96] SA levels decrease as distance to the infection site increases, establishing a gradient where NPR3-NPR1 and NPR4-NPR1 interactions are weakened, so NPR1 accumulates resulting in expression of defence related genes and establishment of SAR. If NPR1 did inhibit PCD this would also explain the lack of PCD in systemic tissues.[96,100]

There is a long history of antagonism between SA and JA signalling pathways that was first established in SA and JA signalling mutants. *At-coi1* mutants show an enhanced basal and induced expression of *PR1* (commonly used as an SA marker gene) while *At-npr1* display enhanced basal and induced expression of *PDF1.2* (commonly used as a JA marker gene).[101,102] Exogenously applied SA will induce JA dependent expression of *PDF1.2* at low concentrations although at higher concentrations the expression is reduced, which suggests that the interaction between the two pathways is dose dependent.[101,102] There are a number of components in the SA signalling pathway that antagonise JA signalling, such as *GRX480* which encodes a glutaredoxin that interacts with TGA transcription factors to regulate expression of *PR* genes. *GRX480* acts downstream of NPR1 and its expression suppresses JA induced expression of *PDF1.2*.[101,103] Overexpression of the transcription factor WRKY70, which acts downstream of NPR1, leads to increased resistance to PstD and *Erwinia carotovora* whereas antisense suppression of WRKY70 caused expression of Vegetative storage protein (*VSP*) and Coronatine induced protein1 (*COR1*).[15] VSP and COR1 are JA induced proteins that could have their JA induced expression repressed by overexpression of *WRKY70*.[15,101] Moreover an increase in SA levels induced *WRKY70* expression whereas an increase in JA levels repressed *WRKY70* expression.[15] From these studies we can see that *WRKY70* is an important integrator of JA and SA antagonism. Another SA signalling component the Elongator complex, which is essential for repression of antioxidant and oxidative stress resistance genes, promotes SA signalling and represses JA signalling.[15,104]

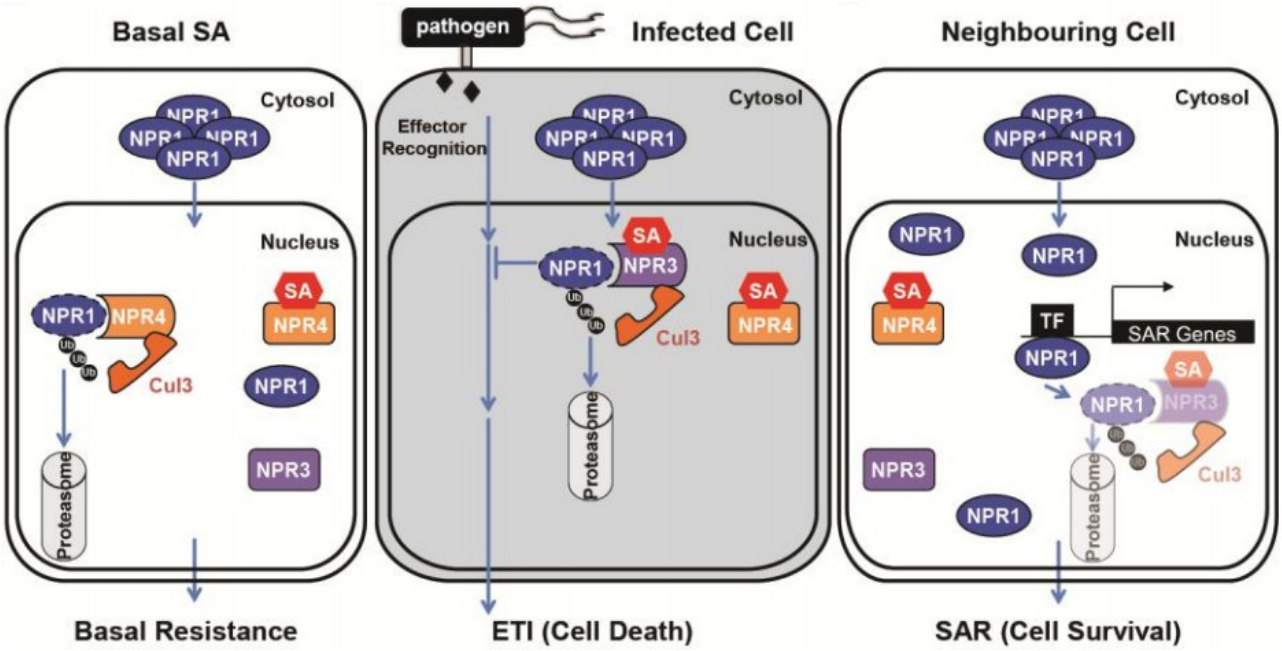


Fig. 8 Model of SA signalling. In the non-induced state (Basal SA) NPR1 oligomers have their intermolecular disulphide bonds disrupted and NPR1 is localized to the nucleus. SA levels are high enough that some NPR4 binding of NPR1 is disrupted but most NPR1 is degraded via NPR4-CUL3 pathway. What NPR1 that remains confers basal resistance. NPR3 cannot bind NPR1 as SA levels are too low. When the Infected Cell recognizes pathogen effectors ETI is triggered and SA levels rise promoting NPR3-NPR1 binding leading to NPR3-CUL3 mediated degradation of NPR1. These conditions result in effector triggered PCD. In Neighbouring cells SA rises but not to the same levels as the cell undergoing ETI so NPR3 and NPR4 binding of NPR1 is limited resulting in NPR1 accumulation and binding of TGA containing transcription factors resulting in SAR.[105]

Much of the work done on SA-JA crosstalk has been focused on SA antagonism of JA but a putative pathway for JA inhibition of SA has been discovered. The NAC transcription factors; ANAC019, ANAC055 and ANAC072, whose expression is partially mediated by JA signalling, have been shown to inhibit SA biosynthesis while increasing SA metabolism (see 1.4.1).[9] There appears to be another pathway for JA mediated antagonism of SA; HDA19 the expression of which is induced by JA/Et.[64] HDA19 deacetylates histones at the *PR1* and *PR2* promoters reducing their expression while loss of function mutants; have increased SA content, increased expression of SA biosynthesis genes, accumulate *PR* genes and have higher resistance to PstD than wild type plants.[106] *At* seems to prioritize SA signalling over JA as preinfection with virulent *P. syringae* or pretreatment with exogenous SA, caused down

regulation of JA mediated resistance and JA marker gene expression upon infection with *Alternaria brassicicola*.[107,108] However, Et signalling changes this prioritization as when the JA/Et pathway is already activated, SA cannot suppress octadecanoid responsive arabidopsis 59 (*ORA59*) mediated expression of *PDF1.2*.[109] This is contrary to SA suppression of ORA59 accumulation when the SA pathway is activated before or at the same time as the JA pathway.[109,110] *Et* plays a further role in SA/JA crosstalk as EIN3 and EIL1 have been shown to repress SID2 which encodes an isochorismate synthase necessary for SA biosynthesis.[111]

1.3 Amino Acids

Amino acids are important compounds containing an amino and a carboxyl group, a lone hydrogen and a variable group bound to a central alpha carbon. Amino acids are the building blocks of proteins, important nitrogen assimilators and precursors for defensive compounds such as camalexin and glucosinalates.[112,113] Amino acids are important regulators of metabolism and plant defence. For instance homoserine or threonine accumulation has been shown to increase plant resistance to oomycete pathogens, glutamine deficiency is correlated with activation of plant defences and expression of SA markers like *PR1* and lysine catabolism produces pipecolic acid which is a regulator of SAR.[114–116]

1.3.1: γ-Aminobutyric Acid

γ-aminobutyric acid (GABA) is a amino acid found in plants, for many years it was thought to be a mere metabolite but recently a more important role for GABA has been established. GABA is involved in the TCA cycle, polyamine metabolism, growth, cell elongation, salt stress and plant resistance to various pathogens.[117–120] The metabolic pathway of GABA is referred to as the GABA shunt (see Fig. 9), which describes the synthesis and breakdown of GABA.[119] GABA is synthesized in the cytosol where glutamate decarboxylase (GAD) breaks down glutamate into GABA in a reaction regulated by intracellular pH and calmodulin.[121–123] GABA is broken down inside mitochondria by GABA transaminase (GABAT) in a reaction dependent on pyruvate and glyoxylate.[119] Interestingly the main source of glyoxylate is from the photorespiratory pathway suggesting that photosynthesis affects GABA metabolism.[124]

The GABAT mediated breakdown produces succinic semialdehyde (SSA) which is turned into succinate by SSA dehydrogenase (SSADH). Succinate is an important compound used in the tricarboxylic acid (TCA) cycle and functions as an electron donor in the mitochondrial electron transport chain.[124]

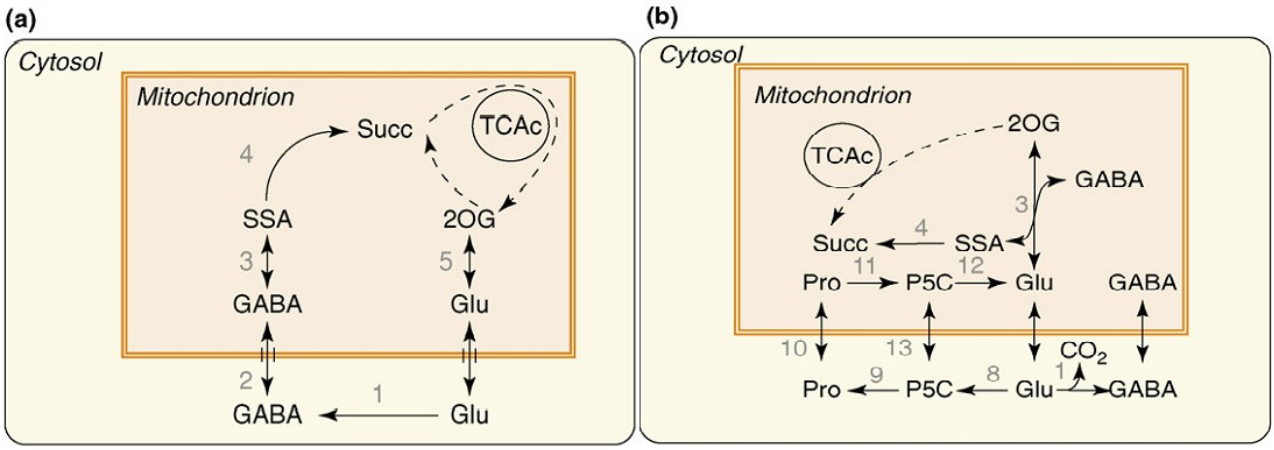


Fig. 9 The GABA shunt. a) Depicts the GABA shunt. Succ is succinate used in the TCA cycle and produces 2-oxoglutarate (2-OG) which can be converted into glutamate (Glu) and recycled in the GABA shunt. (b) The connection of GABA, Glutamate and Proline biosynthetic pathways. Labelled 1; GAD, 2; putative GABA transporter, 3; GABAT, 4;SSADH, 5; Glutamate dehydrogenase (see 1.3.2 for more on 8-13), 8; P5CS, 9; P5CR, 10; mitochondrial glutamate/proline antiporter[125] 11;PDH 12;P5CDH 13; unknown transporter.[119]

There are three well characterised GABA transporters; PROT2, GAT1 and GABP. GAT1 is a proton driven, high affinity GABA transporter that transports GABA across the plasma membrane.[126] In *Nicotiana tabacum* protoplasts GAT1 was shown to localize to the plasma membrane, although it has also been found in chloroplasts and the vacuole, it's localization suggests that it is important for the uptake of GABA from the apoplast.[126] PROT2 and it's family members PROT1 and PROT3 uptake proline and glycine betaine across the plasma membrane with a higher affinity than GABA.[126,127] The three transporters localize the the plasma membrane in *N. tabacum* protoplasts but PROT3 was also present on some internal membranes.[127] The three PROT transporters are differentially expressed with PROT1 expression being highest in the phloem, PROT2 was restricted to the roots (specifically epidermal and cortex cells, although expression was induced in wounded leaves) and PROT3 expression was

restricted to epidermal cells of the leaves.[127] More recently a GABA permease (GABP) was found to transport GABA but not proline in GABA transport deficient yeast, GABP was shown to localize to mitochondria and *gabp* mitochondria had impaired uptake of GABA as well as increased TCA cycle activity.[120] In addition to these transporters there is At5g41800 a poorly characterized transporter protein related most closely to GAT1.[126,128] At5g41800 has been shown to be unable to support *S. cerevisae* growth in media where GABA is the sole nitrogen source but our own previous results (not shown) have shown strong inhibition of PstD growth in *At-at5g41800/gat-1.*[126]

After PtsD inoculation into *At* GABA levels are highly increased compared to inoculations with $MgCl_2$, *GAD1* expression is heavily up regulated although *GAD2* expression is down regulated in PtsD infected plants compared to both mock and PtsD *hrp* infected plants.[129] *GAD1* expression is limited to the roots but *GAD2* is expressed in the leaves, stems, flowers, shoots and roots of *At.*[122,123] PtsD can grow on media where GABA is the sole source of carbon and nitrogen and actually contains three GABAT genes.[130,131] GABA has also been shown to affect the ability of PtsD to grow in *At,* with PtsD growth inhibited in an *At-pop2-1* mutant that accumulates GABA to higher levels than wild type.[130,131] Interestingly growth of PtsD Δ*gabT2/T3/T1* mutants in wild type *At* (ecotype *Landburg erecta*) was inhibited to similar levels as growth of PtsD in *At-pop2-1* mutant plants.[131] The same paper also showed that expression of *hrpL* and *AvrPto* in PtsD was reduced by 50% in media containing 5mM GABA, while the expression of *hrpL* and *AvrPto* was reduced by 90% in PtsD Δ*gabT2/T3/T1*.[131] These results raise the possibility that GABA may have a repressive effect on the coronatine regulon as it is interconnected with the hrp regulon.[131] GABA has also been shown to have an effect on cell elongation, growth and cellular secretions of both organisms which suggests that the GABA mediated repression of growth and secretory pathways may be conserved across *At* and PtsD.[118,131] From this evidence it is clear that GABA plays an important role as a nutrient, stress signal and pathogen growth inhibitor in *At*-PtsD interactions.

1.3.2: Proline

Proline is unique among the proteinogenic amino acids as it contains a second amine group, in plants it accumulates in cells during periods of stress.[132] Proline accumulates under a variety of biotic and abiotic stress conditions, but especially during periods of dehydration (salt stress, drought, freezing).[133] Proline has functions in a variety of

different processes such as adjustment of osmotic potential, stabilization of the cellular structure during dehydration, redox buffering, ROS scavenging and programmed cell death.[133] Proline is synthesized in the cytoplasm under normal conditions but during osmotic stress there is evidence for synthesis in the chloroplasts.[125,133,134] Proline synthesis begins with the reduction of glutamate to glutamate semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS) (see Fig. 10), GSA spontaneously cyclizes to pyrroline-5-carboyxlate (P5C).[125] Pyrroline-5-carboxylate reductase (P5CR) then catalyses the reduction of P5C to proline.[125,135] Proline is catabolized in the mitochondria by proline dehydrogenase (PDH) which oxidises proline to P5C,which is then converted to glutamate by P5C dehydrogenase (P5CDH).[125] There is also an alternative route of proline synthesis where ornithine-delta-aminotransferase (OAT) uses ornithine as a substrate for GSA production, GSA spontaneously converts to P5C which is turned into proline by P5CR.[135]

Proline is mainly moved across the plasma membrane by transporters belonging to three subfamilies of the amino acid transporter (AAC) or amino acid/auxin permease family (hereafter: AAC/AAAP). Amino acid permeases (AAP1-6) function in proton coupled uptake of glutamate, aspartate and proline, lysine-histidine transporter (LHT1 and LHT2) transport neutral and acidic amino acids and proline transporters (PROT1-3) transport proline, glycine betaine and GABA.[135,136] The PROT transporters were covered in depth (see 1.3.1) as they transport GABA as well as proline.

Proline has been shown to accumulate in *At* leaves after infection with avirulent pathogens, while the same incompatible interactions also led to the expression of *P5CS2*, which encodes the rate limiting enzyme P5CS.[135,137] SA is involved in the upregulation of proline biosynthesis as *At* plants with low SA levels do not show enhanced *P5CS2* expression nor do they show increased proline accumulation but *P5CS2* expression and proline accumulation can be stimulated by ROS as well as SA.[137] *P5CS1* expression is induced by an ABA-ABI1 pathway which are strongly induced by PstD infection.[137,138] *ProDH1* and *ProDH2* encode PDH isoforms that accumulate in tissues infected with avirulent PtsD and *PDH* silenced mutants allow for higher levels of growth of PstD *AvrRpm1* compared to wild type plants.[139] Curiously exogenous application of SA activated *ProDH1* expression but even high levels of SA could not induce expression of *ProDH2.*[139] It appears that the expression of *ProDH1* is controlled by SA levels and NPR1 as *At-sid2-2* and *At-npr1-1* mutants were impaired in SA

induced expression of *ProDH1*.[139] The *δOAT* gene that encodes OAT is induced by avirulent pathogens and *At-δoat-1* mutants (*At-prodh1-2* mutants did the same) infected with a non-host pathogen, *Pseudomonas syringae* pv. t*abaci,* supported higher levels of growth than wild type plants.[140] Proline also plays an important role in *Agrobacterium tumefaciens* infection acting as a competitive inhibitor of *A. tumefaciens* 2422-Bra ABC transporter mediated uptake of GABA.[141] GABA stimulates the degradation of a quorum sensing signal which causes a reduction in the number of emerging tumours, which suggests that the success of *A. tumefaciens* infection relies on quorum sensing.[141]

Proline is an important signal during plant-pathogen interactions, especially with avirulent or nonhost pathogens where pathogens trigger HR via ROS signalling.[137,142] This may be due to the induction of proline cycle genes, as a result of ETI and the subsequent SA signalling, which produce ROS via Proline-P5C cycling (see Fig 10).[125,137,142] The increased ROS in the mitochondria stimulate the release of cytochrome c from the mitochondrial membrane which causes PCD and leads to HR response.[140,143]

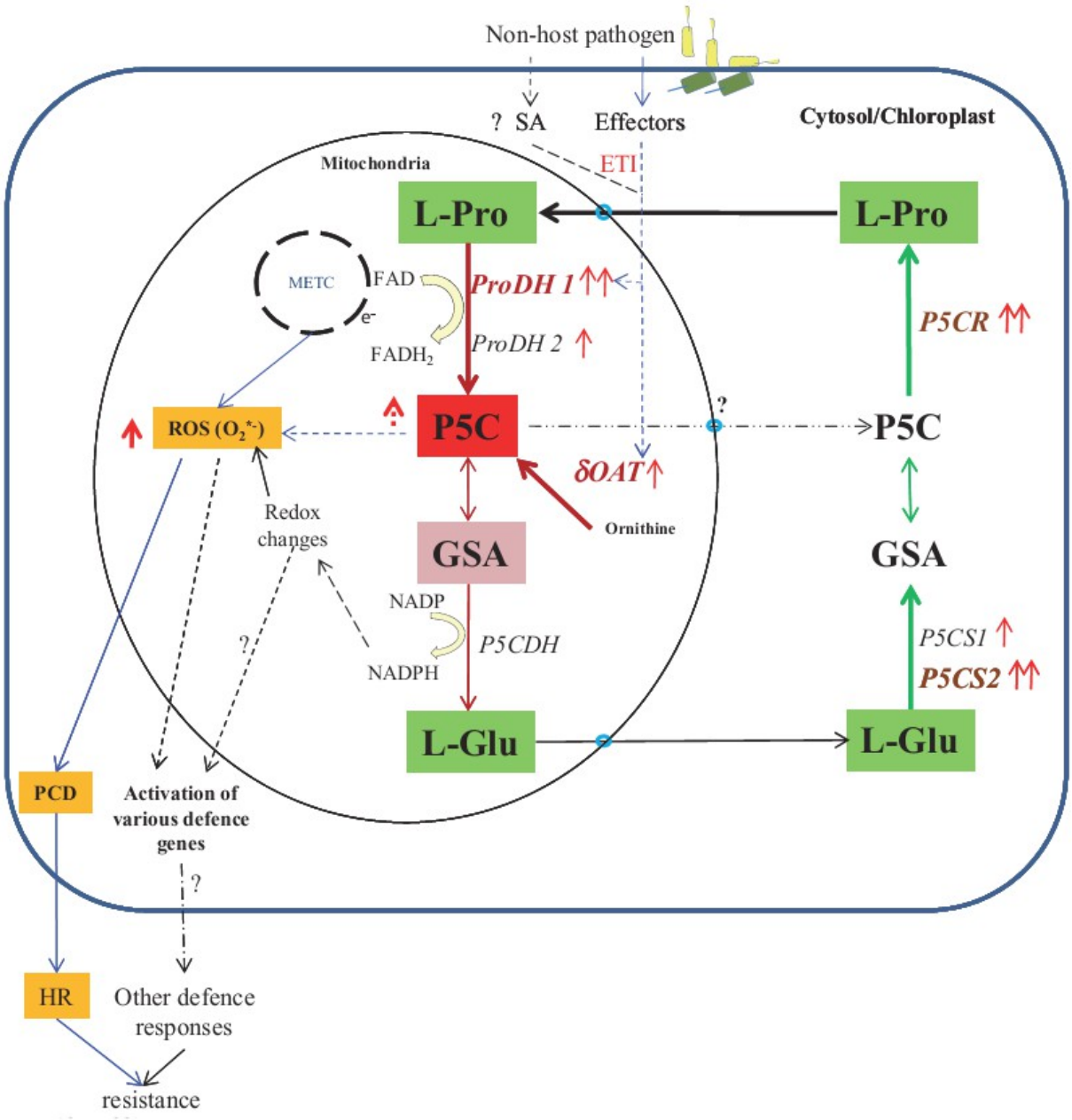


Fig. 10 Model of proline metabolism. Pathogens trigger ETI resulting in increased proline synthesis taking place in the cytoplasm and chloroplasts. Proline is transported into the mitochondria where it is metabolised to P5C by proline dehydrogenases, concomitantly OAT (δOAT in the diagram) metabolises ornithine to P5C. P5C spontaneously converts into GSA which is converted to glutamate by P5CDH, the glutamate is then transported to the cytoplasm where it is converted to proline by P5CS. Increased levels of P5C result in excess P5C being transported to the cytoplasm where it is reduced to proline and transported back to the mitochondria.[140] The PDH-FAD complex mediated oxidation of proline generates electrons for the mitochondrial electron transport chain which leads to generation of ROS which spread to the nucleus and lead to PCD.[140,143]

1.4: Plant Pathogens

1.4.1: *Pseudomonas syringae* pv. *tomato* DC3000

Pseudomonas syringae pathovar *tomato* DC3000 (PstD) is a gram negative hemibiotrophic bacterium, pathogenic on tomato and *At,* that was first isolated in Guernsey in 1960.[144] PstD falls into genomspecies group 3 but is in pathovar group 1.[144,145] PstD invades the apoplastic space, under favourable environmental conditions, through the stomata, hydathodes or wounds whereupon it activates its virulence genes.[145,146] PstD colonizes the apoplastic space where it activates genes to assimilate nutrients found in the apoplast and uses it's type three secretion system (T3SS) to deliver effectors that suppress plant defences and enhance its own fitness.[130] The T3SS is encoded by the *hrp* (hypersensitive response and pathogenicity) cluster of genes and is required for pathogenicity against *At.*[130] Gene expression from the hrp cluster is rapidly upregulated upon infiltration into the plant and in apoplast extracts.[130] As well as the suite of 28 effectors expressed by PstD, it also produces the JA-Ile mimic coronatine.[145,147] The importance of coronatine as a virulence factor is in part due to it's ability to block PAMP mediated defences of stomata and mediate their reopening but it also supports PstD growth, normal symptom development and induces systemic induced susceptibility.[8,9,148] PtsD *cor* mutants (PtsD *cor* is deficient in coronatine biosynthesis) grow to lower levels in wild type plants than PtsD although the growth levels of *cor* mutants is restored in *At* plants deficient in SA accumulation.[8,13,93]

Coronatine is a phytotoxin made of two distinct moieties; coronafacic acid (CFA) which is structurally similar to JA and coronamic acid (CMA).[8] Coronatine promotes PstD virulence in plants by activating a JA-mediated signalling cascade that inhibits SA biosynthesis and promotes SA inactivation.[8,9,148] SA is required for the activation of *PR* genes, PCD and SAR which are necessary for plant defence against biotrophic pathogens.[9] Coronatine acts as a JA-Ile mimic that binds to the COI1-JAZ co-receptor triggering SCF^{COI1}-26S mediated degradation of JAZ proteins, which releases JA responsive transcription factors from JAZ mediated inhibition.[9,35,37] By activating the JA signalling pathway using coronatine PstD is able to antagonize the SA signalling pathway and thus the deployment of biotrophic pathogen specific defence responses. As noted in 1.2.1.5 coronatine is not metabolised by the enzymes that catabolize JA-Ile which would further enhance the ability of coronatine to stimulate JA signalling.[53]

One of these JA responsive transcription factors is MYC2, which upon derepression by coronatine, is able to bind to the G-boxes in the promoter regions of *ANAC019*, *ANAC055* and *ANAC072* causing their expression (see Fig. 11).[9,149] *ANAC019* was shown to bind the promoters of *ICS1*, *SAGT1* and *BSMT1* (*SAGT1* forms SA glucose esters and SA O-β-glucosidase which are inactive storage forms of SA, while *BSMT1* converts SA to inactive methyl SA). Zheng *et al,* 2012 stated that it was reasonable to believe that *ANAC055* and *ANAC072* could also bind these promoters and so repress the expression of *ICS1* and enhance expression of *SAGT1* and *BSMT1*.[9] By repressing *ICS1* which is responsible for SA biosynthesis and enhancing levels of *SAGT1* and *BSMT1* there would be an overall reduction in SA levels. This is supported by SA accumulating to higher levels in *At-anac019-anac055-anac072* (*nac)* plants upon infection with *P. syringae* pv. *maculicola* ES4326 as compared to Col-0 (see 2.1) which shows that the NAC proteins contribute to coronatine mediated suppression of SA levels.[9] Expression of these NACs is also caused by exogenous application of ABA, PtsD induces ABA biosynthesis genes and ABA mediated gene expression which could explain why coronatine deficient *P. syringae* still caused some expression of the NACs.[9] Interestingly *nac* mutants were not deficient in ABA mediated closure of stomata, however coronatine could not mediate their reopening which means that ABA mediated stomatal closure is not dependent on these NAC proteins, although ABA induces expression of these proteins.[9,84] The coronatine mediated induction of the JA signalling pathway also explains why *JAZ* gene expression is induced upon PstD infection and why the expression of most *JAZ* genes is eliminated upon infection with PstD *cor*.[7] It should be noted that JAZ8 degradation is stimulated by coronatine which raises interesting questions about the role of JAZ8 during PstD infection.[36] Induction of the JA pathway also induces expression of *HDA19* which causes a reduction in the expression of *PR1* and *PR2* and lowers expression of genes necessary for the accumulation of SA.[64,106]

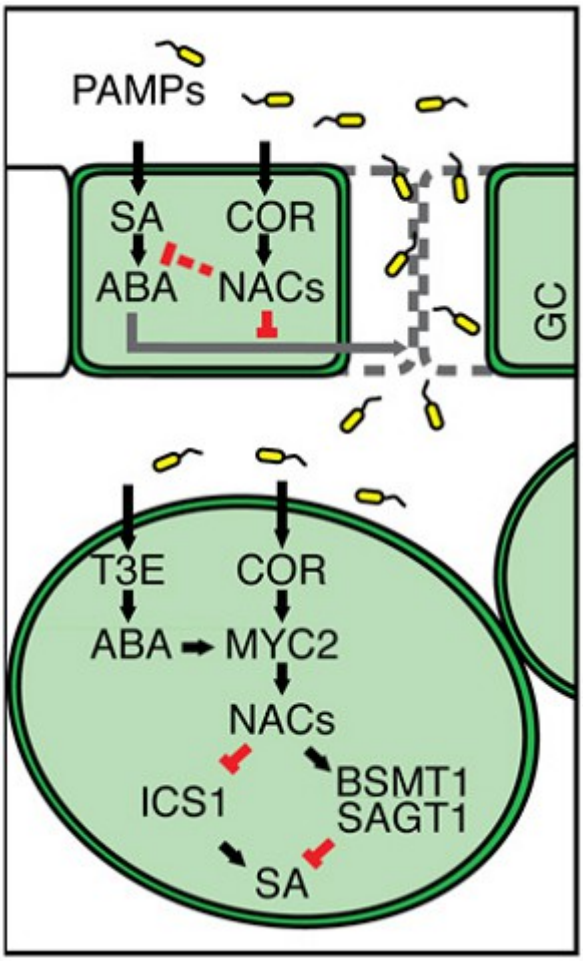


Fig. 11 Coronatine prevents stomatal closure and inhibits SA biosynthesis gene expression. The diagram shows the function of coronatine in guard cells (GC) where upon PAMP perception SA activates ABA mediated closure of stomata. Coronatine induces expression of NAC proteins (*ANAC019*, *ANAC055*, *ANAC072* specifically) through derepression of MYC2, the NACs cause the stomata to reopen allowing more PstD to enter through the stomata. Inside the apoplast type 3 effectors (T3Es) induce ABA biosynthesis causing NAC expression while coronatine (COR) releases MYC2 from JAZ repression leading to expression of NACs. The NACs inhibit *ICS1* expression and upregulate *BSMT1* and *SAGT1* expression, this results in lower levels of SA biosynthesis and higher levels of SA inactivation.[9]

1.4.2: *Botrytis cinerea*

Botrytis cinerea is a polyphagic necrotrophic fungi that causes necrotic lesions in *At*.[150] Through secretion of various compounds and proteins (such as; oxalic acid, botrydial, botcinic acid, cerato-platains, xylanase) that illicit HR/HR like responses (such as ROS generation and SA accumulation) the fungus generate dead tissue for

nutrition.[150–152] In addition to these phytotoxins *B. cinerea* also produces ROS such as hydrogen peroixde and the phytohormones ABA and cytokinin.[151–154]

The importance of the induction of HR as a growth strategy for *B. cinerea* is demonstrated by the suppression of growth in *At-dnd1* a HR deficient mutant, whereas growth of the fungus is enhanced when HR is elicited by avirulent *P. syringae* prior to infection.[155] As JA/Et are the two hormones mainly responsible for defence against necrotrophic pathogens, *B. cinerea* activates HR to provide dead tissue for nutrients while promoting SA signalling which antagonizes JA/Et mediated defences.[151–153] In tomato it was shown that an exopolysaccharide, β-(1,3)(1,6)-D-glucan significantly suppressed the JA-dependent defence markers PI I and PI II and induced SA accumulation.[81,151] A *B. cinerea* sesquisterpene called botrydial induces HR in *At* and induces the expression of two common SA and JA marker genes, *PR1* and *PDF1.2.*[151] Interestingly *At-nahG* and *At-eds5-1*, which accumulate very little SA, were significantly less sensitive to botrydial induced lesions compared to wild type while *At-coi1* was significantly more sensitive than the wild type.[151] This implies that JA modulates sensitivity to botrydial and supports evidence that JA signalling deficient plants are more susceptible to *B. cinerea*.[151] In addition to these examples of phytohormone manipulation the cerato-platanin BcSpl1 induces HR in wild type *At* but a mutation in BAK1, a protein required for detection of PAMPs like flagellin and Ef-Tu, reduced sensitivity to BcSpl1 induced necrosis.[154] It is therefore possible that *B. cinerea* induces PTI to increase SA levels to antagonize JA/Et mediate defences.

Further evidence of the importance of the JA/Et signalling pathway is found in *At* mutants that constitutively express *ERF5* and *ERF6*, two transcription factors able to bind GCC boxes commonly found in the promoter regions of JA/Et responsive genes.[63] Constitutive expression of either *ERF5* or *ERF6* lead to significantly increased resistance to *B. cinerea*, while *At-erf5-erf6* was significantly less resistant.[63] While the constitutive expression of ERF5 or ERF6 lead to increased expression of *PDF1.1* and *PDF1.2a* (two JA/Et regulated defence genes), it also caused significantly reduced expression of *PR-1* (a common SA-induced marker gene) upon UV-C treatment.[63] This evidence suggests that constitutive expression of ERF5/ERF6 caused suppression of SA-mediated gene expression while enhancing JA/Et mediated gene expression which increased *At* resistance to *B. cinerea*.

1.5: Project Aims

The aims for this project were twofold: to look at the role of JAZ proteins in plant defence, and to further characterise the role of amino acid transporter mutants in plant defence.

The role of JAZ proteins in biotrophic and necrotrophic pythopathogen interactions.

- Use a yeast-two-hybrid system to identify novel proteins that interact with JAZ5, JAZ7 and JAZ10, using prey libraries made from early and late stages of *At* infection with PtsD and PtsD *hrpA*.

The expression of JAZ proteins has been shown to be induced by PtsD and *At-jaz10-1* plants show significantly less resistance to PtsD compared to wild type plants.[7] Previous work showed that *At-jaz5/10* plants exhibited more chlorosis in comparison to *At-jaz10* plants suggesting that JAZ5 and JAZ10 act synergistically.

- Investigate whether JAZ mutants are more susceptible to *B. cinerea,* in order to elucidate which JAZs are necessary for resistance to *B. cinerea.*

JAZ mutants combinations were tested for altered susceptibility to *B. cinerea,* to uncover which JAZs are necessary for resistance to *B. cinerea.* JAZs are important regulators of the JA signalling pathway which is important for defence against necrotrophs like *B. cinerea. B. cinerea* actively manipulates the antagonism between SA and JA by producing an exopolysaccharide that activates the SA signalling pathway.[153] Previous work had shown that various *At* JAZ knockout mutants displayed varying susceptibility to *B. cinerea* so we aimed to further these prelimnary findings.

The role of amino acid transporters in biotrophic pythopathogen interactions.

- Continue previous work investigating amino acid transport mutants susceptibilities to PtsD.

Another objective was to continue previous work on amino acid transport mutants that showed varying resistance to PtsD. We have hypothesized that the amino acid content of the varying transport mutants affects the growth of PtsD. It has been shown that PtsD is able to utilise GABA as its sole carbon and nitrogen source and that GABA reduces expression of hrpL and AvrPto in PtsD *gabT* mutants.[130,131]

2: Materials and Methods

2.1: Plant Material and Growth Conditions

Arabidopsis thaliana seed was sown in Levington F2 soil and the pots were incubated at 4°C for two days to vernalize before being transferred to a growth room. Seedlings were pricked out, after the emergence of the first true leaves, into individual pots in a 6x4 tray and placed into a growth room with 10h 100-125 μEinstein light at 22°C and 14h of dark at 20°C.[156] For the first two days in the growth room, clear plastic lids were put on the trays to maintain high humidity. All *A. thaliana* plants used came from the Columbia (Col-0) ecotype including any mutants mentioned herein this study.

Pseudomonas syringae DC3000 and mutants were grown on Kings Broth (with agar 1.5g/100ml KB) plates.[157] The pathogens used in this project were; *P. syringae* DC3000 which contains the empty plasmid pVSP61, *P. syringae* DC3000 *hrpA*- cannot produce a pilus and therefore does not cause disease in *A. thaliana, P. syringae* DC3000 *cor*- that does not produce the bacterial virulence factor coronatine.[13] *P. syringae* DC3000 and mutants were grown on KB plates containing kanamycin (Kan, 50μg/ml) selecting for the pVSP61 plasmid and rifampicin (Rif, 50μg/ml). Spectinomycin (Spec, 10μg/ml) was also used for *P. syringae* DC3000 *cor*- at 28°C.

Botrytis cinerea was cultured on canned apricots in fruit juice from spores stored in glycerol at -80°C. 5μl of thawed spores suspended in glycerol were added to apricots, that had been rinsed in sterile Milli-Q (Millipore) water, on petri dishes. The petri dishes were grown at 22°C until mature spores appeared after 6-7 days.

Saccharomyces cerevisiae (both Y2H Gold and Y187 strains) was grown on Yeast Peptone Dextrose Adenine (YPDA – see Clontech Yeast Protocols Handbook in Appendix 5.1) agar plates at 30°C. All media was prepared according to the Accompanying Yeast Protocol Handbook (Clontech). Transformants were grown on Synthetic defined (SD – see Yeast Protocols Handbook) agar plates lacking Tryptophan (SD/-Trp) or Leucine (SD/-Leu) depending on whether the transformant contained the pGBKT7 or pGADT7 plasmid respectively. Diploid colonies that grew on SD media, lacking in Trp and Leu, containing 5-Bromo-4-Chloro-3-indolyl α-D-galactopyranoside

(X-α-Gal) (40µg/ml) and Aureobasidin A (AbA) (125ng/ml) (SD/-Trp/-Leu/X/A) were plated onto SD/-Ade/-His/-Trp/-Leu/X/AbA media.

Escherichia coli (*E. coli*) was grown on Luria-Bertani (LB) agar plates containing 25µg/ml gentamycin (Gent) at 37°C, or in 10ml LB with 25µg/ml Gent at 37°C with vigorous shaking overnight.[158]

2.2: Determination of *in planta* Bacterial Growth

All PtsD strains were grown in 10ml KB with appropriate antibiotics overnight (Rif, Kan, Spec). The culture was centrifuged at 2000g for 5min before the pellet was resupsended in 10mM $MgCl_2$, this was repeated twice. The suspension was diluted to an OD_{600} 0.2 (1 $x10^5$ colony forming units/ml hereafter cfu/ml) using a spectrophotometer, before undergoing serial 10 fold dilutions to a final OD_{600} 0.0002. The infiltration was performed using the blunt end of a disposable 1ml needleless syringe on the abaxial surface of the *At* leaf. Leaves were nicked by a razor blade and infiltrated either side of the mid vein, ensuring the whole leaf was infiltrated.[84] After the infiltrations the leaves were dried using paper towel. For each *At* genotyping experiments 4-6 plants are infiltrated per *P. syringae* genotype. Col-0 was the wild type control used for all experiments. The plants were then transferred back into the growth room for 3-5 days before being sampled. Three leaves per plant were sampled using a no. 3 cork borer (8mm diameter). Three samples from a plant were added to a 2ml microfuge tube containing 1ml 10mM $MgCl_2$ then lysed in a tissue lyser (Qiagen) for 2 x 1min at 24Hz using a tungsten ball bearing. Each sample was then diluted to 10^{-1}, 10^{-2}, 10^{-3} the original concentration and 6 x 10µl of each concentration was plated on KB plates containing rifampicin (50µg/ml) and kanamycin (50µg/ml). The plates were left to grow at 28°C until two dilutions are countable, this is normally ~20h . The number of colonies per 10µl spot were counted in two dilutions and the log cfu/ml was calculated. Student's t-test was used to determine whether there was any significant ($p \leq 0.05$) difference in bacterial growth between the sample and the control.[159]

2.2.1: GABA Co-Inoculations

Population experiments performed with *P. syringae* genotypes and GABA were performed as described above, except that the inoculums used are as follows. OD_{600}

0.0002 PtsD in $MgCl_2$, OD_{600} PtsD in $MgCl_2$ with 10mM GABA treatment and OD_{600} PtsD in $MgCl_2$ with 1mM GABA. After infiltration the plants were returned to the growth room for 4 days before being harvested as described above.

2.3: Phenotypes

Phenotyping of different *A. thaliana* mutants was done by infiltrating 3-4 leaves per plant (as described above) with a suspension of *P. syringae* in 10mM $MgCl_2$ with an OD_{600} 0.002. The plants were left for 3-5 days before being photographed.[84]

Phenotyping with *B. cinerea* was done by growing the *B. cinerea* to obtain mature spores (as described above - see 2.1); 3ml of sterile water was added to the petri dish and the spores were resuspended using a glass rod. The spore suspension was filtered through a sterile piece of miracloth into a 50ml falcon tube and spun at 2000g for 5min. The pellet was resuspended in 2ml 50% grape juice. The spores were counted using a hemocytometer and the solution diluted with 50% grape juice until there was 1 x 10^5 spores/ml. To infect the plants 3-4 leaves per plant were cut at the petiole so there was approximately 5mm of petiole attached to the leaf. These leaves were then placed on 0.8% agar plates and had a 5µl drop of the spore solution placed on the adaxial surface either side of the mid rib. The plates were left for 3 days before being photographed. The lesion sizes were determined from photographs taken of the leaves using ImageJ (http://rsb.info.nih.gov/ij/), significant differences were determined using the Student's t-test.

2.3.1: Genotyping Amino Acid Transporter Mutants

We used PCR to confirm that our amino acid transport mutants were homozygous knockouts using the primers in the Table 1 below. The DNA was obtained using a "Shorty buffer" extraction method as follows; one leaf per plant being genotyped was removed using clean scissors and placed in a 2ml microfuge tube. 500µl Shorty buffer was added along with a tungsten ball bearing.[160] Tissue was disrupted using a tissue lyser for 2 x 1min at 24Hz. 500µl phenol-chloroform was added to the tube and was briefly vortexed before being centrifuged at 13000g at room temperature for 5min. After centrifugation the upper phase (normally ~450µl) was removed and transferred into a 1.5ml microfuge tube. An equal volume of isopropanol was added before the sample was centrifuged at 13000g at room temperature for 10min. All the liquid was then

decanted from the microfuge tube before the tubes are centrifuged briefly again so that any remaining liquid can be pipetted away without disturbing the pellet. The pellet was resuspended in 100µl sterile water before freezing and storage at -20°C.

The primers used were designed by Bing Zhang a former PhD student apart from LBb1.3 whose sequence was provided by SALK (http://www.salk.edu/). The reactions were run with the following reagent concentrations;

1x Phusion Buffer (New England Bioscience) (from 5x)
200µM dNTPs
0.5µM primers (for both forward and reverse primers)
1pg-10ng DNA (for these reactions we used 2µl of the 100µl DNA extraction described above)
0.02U/µl Phusion polymerase (New England Bioscience)

The reactions were performed under the following conditions; 98°C hot start 30s, 35 cycles of; 98°C for 10s, 56°C for 30s, 72°C for 1min. 72°C for 10min. The primers used are in Table 1 below.

Primer Name	Sequence
gat-1 KO F	ATATCGGCACCAGTGAAAGG
gat-1 KO R	CAACGATTGTAGCCATCACG
At5g41800 KO F	GATGTGGTGCAGGATCTGG
At5g41800 KO R	ACGTTTAGGAGCGTTTTTCG
prot-1 F (SALK_030711_RP)	TCAACAAGGTAGCTTCGACAAC
prot-1 R(SALK_030711_LP)	GAAATTGGGGAAACAATCTCC
LBb1.3	ATTTTGCCGATTTCGGAAC

Table 1. Sequences of primers used for genotyping amino acid mutants.

2.4: Mass Spectrometry

At plants were inoculated with OD_{600} 0.15 PtsD in the same manner as described above in 2.2, although control plants were not inoculated. After the prescribed amount of time had passed, 3 leaves per plant were cut and pooled with 3 leaves from another plant (same genotype) in an aluminium foil packet. This packet of 6 leaves constituted one sample. The samples were immediately passed to liquid nitrogen. The samples were transferred to a -80°C freezer until all time points were harvested. Samples were freeze

dried for two days. The leaves from each sample were passed into 2 ml microfuge tubes containing a tungsten ball bearing and placed into a tissue lyser (Qiagen) and lysed for 2x1min at 24Hz. 10mg of each sample was weighed out and placed into 1.5ml microfuge tubes, the precise weights were always recorded within a 0.2mg margin of error. 400µl of extraction buffer was added to each sample, the samples were vortexed until all the sample was suspended.

Extraction buffer:

10% methanol
1% acetic acid in H_2O
2µl 24µM Jasmonic acid per sample
1µl 24µM Salicylic acid per sample
2µl 24µM Abscisic acid per sample

Samples were incubated on ice for 30min before being vortexed briefly and sonicated for 10min at ≥8°C. The samples were then centrifuged at 13000g for 10min at 4°C. The supernatant was carefully removed and transferred to a clean microfuge tube and a second extraction was performed on the pellet using 400µl of extraction buffer (containing no internal standards). The supernatant from the second extraction was added to the first and vortexed to mix. This solution was then filtered using a blunt tipped syringe through a 4-SF-02(PV) Chromacol filter.

The resulting solutions were analysed using mass spectrography by Dr. Venura Perera (v.perera@exeter.ac.uk) using the method described in *Forcat et al,.* 2008.[161]

2.5: Yeast Two Hybrid System

The yeast-two-hybrid is a system by which it possible to investigate protein-protein interactions *in vivo*. The system works by selecting a bait proteins which are proteins whose interactions with other proteins you will investigate. The prey proteins are proteins whose interactions with the bait proteins you will test. In this project our bait proteins are JAZ5, JAZ7, JAZ10.1, JAZ10.3 and JAZ10.4. We have selected these proteins as they are important regulators of the interaction between PstD and *At*. The prey proteins that we used in this project were comprised of three cDNA libraries taken from *At* infected with PtsD and PtsD *hrpA*. The bait and prey proteins are inserted into two different plasmids pGBKT7 and pGADT7 respectively. These plasmids contain a

nutritional selectable marker (tryptophan and leucine autotrophy genes respectively) and antibiotic resistance (for resistance to kanamycin and ampicillin respectively) as well as GAL4 activation and binding domains (GAL4 AD and GAL4 BD). When bait and prey proteins are expressed as fusions with GAL4AD and GAL4BD *in vivo,* interactions between bait and prey proteins will cause GAL4AD and GAL4BD to be brought close enough to interact and bind to GAL4 promoters. The GAL4 promoters in this yeast-two-hybrid system control expression of four "reporter genes". The expression of these reporter genes is controlled by three hetrologous GAL4 promoters so only successful bait-prey interactions can activate their expression. The reporter genes in this system are: *AUR1-C* that confers resistance to Aureobasidin A (AbA), *HIS3* that allows histidine biosynthesis, *ADE2* that allows adenine biosynthesis and *MEL1* that allows for α-galactosidase biosynthesis. The reporter genes allow for the growth of yeast on media containing Aureobasidin A and lacking histidine and adenine (DDO/X/A). α-galactosidase is secreted by yeast cells causing them to turn blue in the presence of X-α-Gal (see 2.1). The reporter genes allow us to control for successful protein-protein interactions by mating yeast containing our bait proteins with yeast containing our prey cDNA library. By spreading the resulting diploids on media containing AbA and X-α-Gal but lacking in tryptophan and leucine we can select colonies with bait-prey protein interactions. These colonies are then spread onto higher stringency media which also lacks histidine and adenine, any colonies that survive on this media contain true bait-prey interactions. It is then possible to rescue the prey plasmid and sequence it so the prey protein can be identified.

We were supplied with three PD207 vectors containing full length *JAZ5*, *JAZ7* and *JAZ10* sequences which we transformed into *E. coli* (see 2.5.1) to amplify. Plasmids were purified by QIA prep Spin miniprep kit. We checked the product for the presence of the JAZ inserts using PCR and gel electrophoresis (see 2.5.2). We used high fidelity Taq to amplify and ligate 15bp extensions to the '5 ends of our JAZ sequences in accordance with the In Fusion HD manual (see 2.5.3). These extended JAZ sequences were recovered using the Qiaquick Gel Extraction kit. We linearised the pGBKT7 vector using BamH1 and EcoR1 (see 2.5.5) as to be able to ligate our JAZ sequences into pGBKT7 using the In Fusion HD system. We purified the linearized pGBKT7 using the Qiaquick Gel Extraction kit and performed a fusion PCR to ligate our JAZ sequences into the pGBKT7 vector using the In Fusion HD cloning system (see 2.5.4).

The pGBKT7-JAZ constructs were then transformed into *E. coli* (see 2.5.1) and plated on LB plates. Six colonies from each set of transformations were checked for the JAZ inserts with colony PCR (see 2.5.7). After confirmation of the presence of pGBKT7-JAZ constructs, the four plasmids were purified using the QIAprep Spin miniprep kit (Qiagen). Each of the constructs was then sequenced (see 2.8.1) and checked via enzyme digestion (see 2.5.5). The bait vectors were then transformed into *S. cerevisae* Y2HGold (Y2HG) provided in the Matchmaker Gold Yeast Two Hybrid System (Clontech) using the Yeastmaker 2 protocol (Clontech - see Appendix 5.1). To check that the JAZs were being correctly expressed we performed a western blot on the transformed Y2HG against the Gal4 BD tag (see 2.5.8).

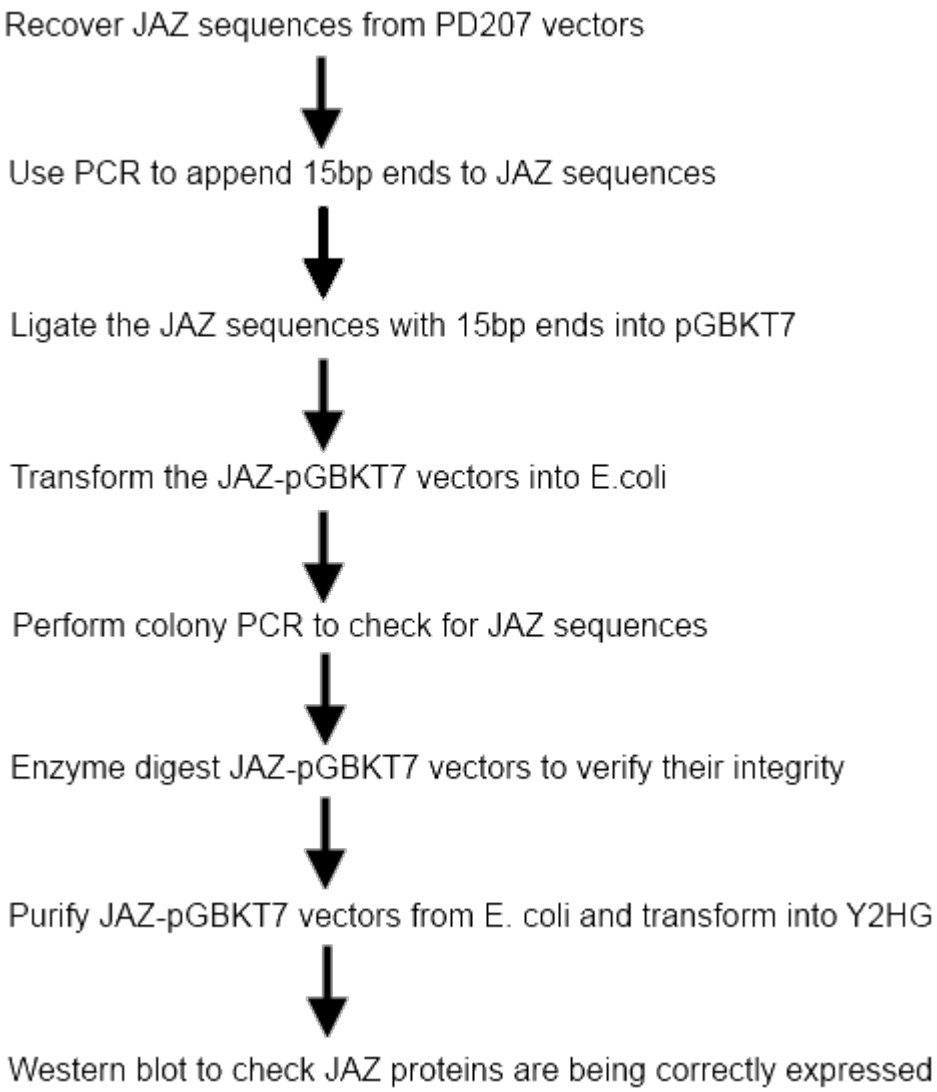


Fig. 12 Flow diagram of steps described in 2.5 to create yeast-two-hybrid bait proteins.

2.5.1 Transformation of *E. coli*

PD207 vectors containing *JAZ5*, *JAZ7*, *JAZ10.1* and *JAZ10.3* were transformed into competent *E. coli* DH5α cells using heat shock. 100µl of competent cells was mixed with 1-5ng of plasmid DNA and incubated in an ice bath for 30min. The mixture was heat shocked at 42°C for 30 seconds and immediately passed to ice, 1ml LB was added and the cells were incubated at 37°C with vigorous shaking for 1h. The cells were plated on LB agar plates with appropriate antibiotics (see 2.1). The plasmids were then recovered using the QIAprep Spin miniprep system (Qiagen) so that the JAZ sequences could be PCR amplified out and have the In Fusion HD ends annealed to them.

2.5.2: Polymerase Chain Reaction of JAZ Sequences in PD207

PCR was used to verify that the JAZ sequences from PD207 vectors had been successfully recovered. These PCR reactions were run using a Phusion Hot Start polymerase (NEB). Reaction volumes used were 20µl and 50µl, final concentrations of reagents were as follows;
1x Phusion Buffer (New England Bioscience) (from 5x)
200µM dNTPs
0.5µM primers (for both forward and reverse primers)
1pg-10ng DNA
0.02U/µl Phusion polymerase (New England Bioscience)
We performed gradient PCR with temperature cycles as follows; 98°C hot start 30s, 35 cycles of; 98°C for 10s, 60.3-69.4°C for 15s, 72°C for 25s. 72°C for 10min. The products of the reaction was then electrophoresed on a 1.2% agarose gel and visualized using ethidium bromide. The primers used are in the Table 2 below.

2.5.3: Ligation of Specific Ends to JAZ Sequences

We designed primers with a 15bp homology at the '5 end to the ends of the linearised pGBKT7 vector. These primers were used in a PCR reaction to anneal specific ends to JAZ sequences for use with the In Fusion HD kit (Clontech). These primers can be found Table 3 below. These PCR reactions were run using Phusion Hot Start polymerase (NEB). We used a 20µl reaction volume, final concentrations of reagents were as

follows;

1x Phusion Buffer (New England Bioscience) (from 5x)

200µM dNTP

0.5µM primers (for both forward and reverse primers)

1pg-10ng DNA

0.02U/µl Phusion high fidelity polymerase (New England Bioscience)

We performed gradient PCR with temperature cycles as follows; 98°C hot start 30s, 35 cycles of; 98°C for 10s, 60.3-69.4°C for 15s, 72°C for 25s. 72°C for 10min. The products of the reaction were electrophoresed on a 1.2% agarose gel and visualized using ethidium bromide.

Primer Name	Sequence
JAZ5FP	ATGTCGTCGAGCAATGAAAATGCTAAG
JAZ5RP	CTATAGCCTTAGATCGAGATCTTTCG
JAZ7FP	ATGATCATCATCATCAAAAACTGCGAC
JAZ7RP	CTATCGGTAACGGTGGTAAGG
JAZ10FP	ATGTCGAAAGCTACCATAGAACTC
JAZ10.1RP	TTAGGCCGATGTCGGATAGTAAG
JAZ10.2/3RP	TTACCTCTCCTTGCGCTTCTC
JAZ10.4 RP	CTAATCTCTCCTTGCGCTTCTCGA

Table 2. The primer sequences used to PCR JAZ sequences out of PD207 vectors.

Primer Name	Sequence
JAZ5FP	CATGGAGGCCGAATTCATGTCGTCGAGCAATGAAAATGCTAAG
JAZ5RP	GCAGGTCGACGGATCCCTATAGCCTTAGATCGAGATCTTTCG
JAZ7FP	CATGGAGGCCGAATTCATGATCATCATCATCAAAAACTGCGAC
JAZ7RP	GCAGGTCGACGGATCCCTATCGGTAACGGTGGTAAGG
JAZ10FP	CATGGAGGCCGAATTCATGTCGAAAGCTACCATAGAACTC
JAZ10.1RP	GCAGGTCGACGGATCCTTAGGCCGATGTCGGATAGTAAG
JAZ10.2/3RP	GCAGGTCGACGGATCCTTACCTCTCCTTGCGCTTCTC
JAZ10.4RP	GCAGGTCGACGGATCCCTAATCTCTCCTTGCGCTTCTCGA

Table 3. The primer sequences of primers used to ligate ends specific to linearised pGBKT7 onto our JAZ sequences.

2.5.4: In Fusion HD Cloning Reaction

We recovered our linearised pGBKT7 vector and JAZ sequences with 15bp homology to the ends of the linearised pGBKT7 vector using the Qiaquick gel extraction kit. We used the In Fusion HD cloning system (Clontech) to ligate the JAZ sequences into the pGBKT7 vector. The reaction mixture used was as follows:

10-200ng purified PCR fragment
50-200ng linearised vector
2µl 5x In-Fusion HD enzyme premix
Sterile deionized water is added up to 10µl total reaction volume

The reaction is mixed and incubated at 50°C for 15min. The reaction is immediately placed on ice thereafter it can be stored at -20°C or be used for transformations as described in 2.4.6.

2.5.5: Enzyme Digestion

Following manufacturers instructions (see Matchmaker Gold Yeast Two Hybrid manual see Appendix 5.1) pGBKT7 was linearised using EcoR1 and BamH1 for 1h at 37C. Digestions were checked on an agarose gel. The reagents in the 100µl reaction volume were as follows;

10µl buffer 3 (10U/ml) (New England Bioscience)
1ul Bovine serum albumin
13µl pGBKT7 (500ng/µl)
Sterile deionized water is added up to 100µl total reaction volume

We added either 0.5µl (20U/µl) EcoR1 or 1µl (10U/µl) BamH1 and incubated the reactions at 37°C for 1h. After confirming that digestion had taken place using agarose gel electrophoresis we added 0.5µl (20U/µl) EcoR1 and 1µl (10U/µl) BamH1 to the BamH1 and EcoR1 digested samples respectively and incubated them at 37°C for 1h. After confirmation of successful digestion using agarose gel electrophoresis, we loaded the remaining 94µl of the reaction mixtures into a 1.2% agarose gel and electrophoresed the samples at 80V. We used the Qiaquick Gel Extraction kit to purify the digested pGBKT7 and used a Nanodrop (Agilent) to check the yield.

JAZ constructs were checked for integrity via enzyme digest. The restriction enzymes that the constructs were treated with are in the Table 4 below.

Construct	Restriction Enzymes used	Size of band expected (bp)
pGBKT7-JAZ5	BamH1/EcoR1	7305, 830
pGBKT7-JAZ5	Nco1	7373, 762
pGBKT7-JAZ7	BamH1	7622, 135
pGBKT7-JAZ7	BamH1/EcoR1	7305, 317, 135
pGBKT7-JAZ10.1	BamH1/EcoR1	7904, 610
pGBKT7-JAZ10.3	BamH1/EcoR1	7868, 574
pGBKT7-JAZ10.4	BamH1/EcoR1	7305, 509
pGBKT7-JAZ10.1	EcoR1/ClaI	7904, 610
pGBKT7-JAZ10.3	EcoR1/ClaI	7868, 574
pGBKT7-JAZ10.4	EcoR1/ClaI	7305, 509
pGBKT7-JAZ10.1	Xba1	3940, 2992, 988
pGBKT7-JAZ10.3	Xba1	3940, 2992, 988
pGBKT7-JAZ10.4	Xba1	6842, 988

Table 4. The restriction enzymes used to cut pGBKT7-JAZ plasmids and the size of the bands expected upon visualization of the reactions.

The reactions mixtures were made as follows:
19.75µl sterile water
2µl pGBKT7-JAZx construct (DNA concentration was between 500ng/µl and 750ng/µl)
2.5µl 10x buffer (New England Bioscience buffer)
0.25µl 100x BSA (except for those reactions containing Nco1)

For each reaction 0.5µl enzyme was used, except for Xba1 where 2µl was used as there were more restriction sites for Xba1. The digestions took place at 37°C for 1h. The 10x buffer refers to a numbered NEB buffer. The specific buffers required for each enzyme are in the Table 5 below.

Samples were digested for 1h and 10µl of each reaction was loaded onto a 1.2% agarose gel and visualized with ethidium bromide. For pGBKT7-JAZ7 digestions and pGBKT7-JAZ10 digestions with EcoR1/ClaI small fragments (<250bp) were expected so 17µl of each reaction mixture was loaded instead. The reactions were run on a 1.2% agarose gel at 75V for 1h and visualized with ethidium bromide.

Enzyme	Buffer required
Nco1 (20U/µl)	Buffer 3
BamH1 (10U/µl)	Buffer 3
BamH1/EcoR1 (10U/µl and 20U/µl)	EcoR1 buffer
ClaI/EcoR1 (5U/µl and 20U/µl)	Buffer 4
Xba1(12U/µl)	Buffer 4

Table 5. The NEB buffers required for different restriction enzymes used in the restriction analysis of pGBKT7-JAZ plasmids.

2.5.6 Yeast Transformation

We performed all yeast transformations according to the Yeastmaker manual (Clontech – see Appendix 5.1) which describes the creation of competent cells using lithium acetate (LiAc), followed by polyethylene glycol(PEG)/LiAc transformation. A fresh colony of yeast (Y2HGold for bait, Y187 for prey) was inoculated into 3ml YPDA in a sterile 15ml universal and incubated at 30°C with 250rpm shaking for 8-12h. 5µl of the culture was transferred to 50ml YPDA in a 250ml flask and incubated at 30°C with 250rpm shaking until the OD_{600} is 0.15-0.3 (~16-20h). The culture was centrifuged at 700g for 5min at room temperature, the pellet was resuspended in 100ml YPDA. The culture was incubated at 30°C with 250rpm shaking until the OD_{600} is 0.4-0.5 (~3-5h). The culture was divided into two 50ml falcon tubes and centrifuged at 700g for 5min at room temperature. The pellets were resuspended in 30ml sterile water before centrifugation and resuspension in 1.5ml 1.1x TE/LiAc buffer. The cultures were transferred to 1.5ml microfuge tubes and centrifuged at max speed (13000g) for 15s and the pellet was resuspended in 600µl 1.1x TE/LiAc.

100ng of plasmid DNA was combined with 50µg denatured carrier DNA in a 1.5ml microfuge tube. The competent cells were added and the contents of the microfuge tube were gently mixed. 500µl PEG/LiAc was added and the mixture was incubated at 30°C for 30min, with gentle mixing every 10min. The microfuge tube was centrifuged at maximum speed (13000g), the pellet was resuspended in 1ml YPD Plus. The cells were incubated at 30°C with shaking for 90min before the cells were pelleted by high speed centrifugation. The pellet was resuspended in 1ml 0.9% NaCl solution.

100µl of a 1/10 and 1/100 dilution of the 1ml suspension was plated onto appropriate selective SD plates (pGBKT7 confers -Trp autotrophy while pGADT7 confers -Leu

autotrophy) and incubated at 30°C for 3-5 days.

2.5.7: Colony Polymerase Chain Reaction

Six discrete yeast colonies were randomly selected from SD media plates using a 10µl pipette tip and were suspended in 10ul water and heated to 98°C for 5min. A total reaction volume of 50µl was made up using:

10µl denatured yeast cells
23µl sterile water
1.5mM $MgSO_4$
200µM dNTP
0.5µM primer (both forward and reverse)
1.5µl Taq polymerase

The resulting product was visualized on 1.2% agarose gel with ethidium bromide.

Colony PCR on pGBKT7-JAZ constructs were run at 98°C 30s, 35 cycles of; 98°C for 10s, 56°C for 30s, 72°C for 1min, 72°C for 10min using primers found in 2.5.3.

Colony PCR on Y187 containing putative interactors were run at 94°C 2min, 35 cycles of; 98°C for 30s, 55°C for 30s, 72°C for 1min. 72°C for 10min using primers found in 2.8.2.

2.5.8: Western Blot

We used the protocol for protein extraction described in the Accompanying Yeast Protocol handbook (Clontech). Briefly; we grew our transformed strains to OD_{600} 0.4-0.6 whereupon we centrifuged the sample and snap froze the pellet in liquid nitrogen. A protein extraction was performed on the pellet using the Urea/SDS method and the western blot was performed using these extracts.[162]

Protein samples were run on a 1% SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) gel to size separate the protein samples using Positope protein (Invitrogen) and protein extracted from Y2HG-pGBKT7-53 as controls. We used a resolving and stacking gel whose composition was as follows;

Resolving Gel:

5ml 1.5M tris(hydroxymethyl)aminomethane (Tris) pH8.8

200µl 10% SDS
5ml Acrylamide/Bis-acrylamide (Acryl/bis)
9.8ml H_2O

Stacking Gel:

2.5ml 0.5M Tris pH6.8
100µl 10% SDS
1.2ml Acryl/Bis
6.2ml H_2O

We used 100µl ammonium persulphate mixed with 10µl Tetramethylethylenediamine to polymerize Acryl/Bis.

Protein samples were boiled for 5min before 20µl of each sample and 5µl positope was loaded into the gel and run at 200V until the samples began to leak out the bottom of the gel (~1h). Empty wells were filled with cracking buffer from the Urea/SDS protein extraction. We always ran two gels, one for western blotting and the other for coomasie staining. The coomasie stained gel was incubated with coomasie staining buffer and gently agitated for 30min. The staining buffer was discarded and the gel was covered with destaining buffer and gently agitated for 30min.

While the SDS/PAGE gels are running, Towbin buffer was prepared from a 10X stock[163]:

100ml 10X Towbin
200ml methanol
700ml H_2O

Filter paper was soaked in 1X Towbin for 5min, a Polyvinylidene fluoride (PVDF) membrane was soaked in methanol for ~15 seconds, before being soaked in H_2O for 5min and then 1X Towbin buffer for 10min. The filter paper, PVDF membrane and the gel were assembled as follows; 3 layers of filter paper, PVDF membrane, gel, 3 layers of filter paper. This "sandwich" was then clamped tightly together and placed into a transfer tank and covered in transfer buffer with the PVDF membrane nearest the anode and the gel nearest the cathode. The transfer was run at 100V for 1h at 4°C.

Once the transfer was complete the PVDF membrane was blocked in Phosphate buffered saline (PBS), 0.05% Tween20 (Sigma Aldrich) and 5% skim milk for 1h at

room temperature with slight agitation. The membrane was washed three times in 20ml PBS with 0.05% Tween20 (PBST) for 10min then incubated with 1:5000 Anti-myc (Invitrogen) antibody in 10ml PBST with 5% skim milk for 2h. We used an anti-myc antibody as our bait proteins are expressed as a fusion with a MYC tag from the pGBKT7 plasmid. We washed the membrane three times in 20ml PBST for 10min, before incubating the membrane with goat anti-mouse HRP 1:5000 with 10ml PBST with 2.5% skim milk. The PBST wash was repeated and then the blot was treated with enhanced chemiluminesence (ECL). We used 0.05ml/cm^2 ECL reagents for our blots and captured the chemiluminesence using x-ray film.

2.5.9: Control Experiments

As described in the Matchmaker Gold Yeast Two Hybrid System user manual, three control experiments must be performed on the bait before screenings can occur. The three experiments are control of autoactivation of reporter genes, bait toxicity and protein expression.

Control of autoactivation was performed by transforming pGBKT7-JAZ constructs into Y2HG (see 2.5.6) and plating 100µl of a 1/10 and 1/100 dilution of the product of the transformation onto SD/-Trp, SD/-Trp/X and SD/-Trp/X/A plates. The plates were incubated at 30°C for 3-5 days after which there should be distinct colonies on SD/-Trp and SD/-Trp/X but none on SD/-Trp/X/A plates.

Bait toxicity was determined by transforming Y2HG cells with empty pGBKT7 and with pGBKT7-JAZ. 100µl of 1/10 and 1/100 dilutions are plated onto SD/-Trp plates and the plates were incubated at 30°C for 3-5 days. There should be no significant difference in colony size between the two transformants.

To determine whether the yeast was correctly expressing our JAZ inserts we performed a western blot (see 2.5.8)

2.6: Construction of the cDNA library

2.6.1: RNA Species Creation

We used RNA obtained from two separate time course experiments to create three cDNA libraries. Firstly we pooled RNA from At infected with PtsD with RNA from At

infected with PtsD *hrpA*- (known as the Mixed library). The RNA used was extracted 2, 3 and 4 hours post infection (hpi). We selected 5 samples per time point, using 1µl of each sample and pooling them. Secondly we pooled 1µl RNA from 8, 10, 11 and 12hpi, sampling five samples per time point of the PtsD time course(known as DC3000 library). We then did the same for the PtsD *hrpA*- time course (known as hrpA library). The concentration of the three pooled RNA samples was checked using a Nanodrop (Agilent) before 2µg of each samples was run on a 1.5% agarose/formaldehyde denaturing gel to check the integrity of the RNA. The denaturing buffer was made up of:

400µl formamide
120µl formaldehyde
100µl 10x MEN buffer:

200mM MOPS
50mM sodium acetate
20mM EDTA
H_2O to bring volume to 1l
Bring to pH7 with NaOH

The gel was made of:

0.75g agarose
5ml 10x MEN
8ml formaldehyde
37ml H_2O

2µg of each sample was denatured for 10min at 65°C in 8.7µl of a denaturing buffer and sterile water up to 14µl, with ethidium bromide added, before being passed to ice immediately. The RNA was vortexed and centrifuged before being loaded onto the gel. The gel was run at 60V until the RNA had migrated about ¾ of the way down the gel.

2.6.2: cDNA First Strand Synthesis

The Mate & Plate Library system (Clontech) was used to construct our cDNA library. First strand synthesis on the comprised;

2.0µg total RNA
1µl CDS III primer

1-2µl H_2O to make a total reaction volume of 4µl.

The reaction was incubated at 72°C for 2min before incubation on ice for 2min and centrifugation at 14,000g for 10s. The 4µl reaction was added to a mixture of:

2µl 5x First-Strand buffer
1µl 100mM DTT
1µl 10mM dNTP
200U/µl SMART MMLV Reverse Transcriptase

The 9µl reaction was incubated at 42°C for 10min, 1µl SMART III-modified oligo mix was added and the reaction mixture was incubated at 42°C for 1h. The reaction was heated to 75°C for 10min, allowed to cool to room temperature before 1µl Rnase H was added and the reaction is heated to 37°C for 20min. The reaction can be stored at -20°C or used immediately for Long Distance Polymerase Chain Reaction.

2.6.3: Long Distance Polymerase Chain Reaction

As described in the Mate & Plate Library system manual (see Appendix 5.1) the product of the first strand synthesis was used in a LD-PCR to amplify the first cDNA strand created during first strand synthesis. Two 100µl PCR reactions were set up using 2µl of the first strand synthesis reaction for each 100µl reaction. Each 100µl reaction is composed of;

70µl H_2O
10µl 10x Advantage 2 PCR buffer
2µl 10mM dNTP mix
2µl 10µM 5' PCR Primer
2µl 10µM 3' PCR Primer
10µl 10x Melting Solution
2µl 50x Advantage 2 Polymerase mix
2µl first strand synthesis reaction

The PCR was programmed as follows; 95°C 30s, 25 x 95°C 10s 68°C 6min, 68°C 5min. The PCR was programmed so that every cycle the extension time increased by 5 seconds. 7µl of the reaction was checked on a 1.2% agarose gel with 0.25µg of 1kb DNA ladder visualized with ethidium bromide. The reaction can be stored at -20°C until it is purified in a column.

2.6.4: cDNA Purification and Precipitation

The product of LD-PCR was size fractionated using a Chroma Spin TE 400 column (Clontech), before the cDNA was ethanol precipitated. The column was inverted until the gel matrix was homogenous and placed into a 2ml collection tube. The column was centrifuged using swing bucket rotors at 700g for 5min to purge the buffer from the column. The collection tube was discarded and the column was placed into a new collection tube. 93µl of the reaction mixture from LD-PCR was pipetted into the column. The column was centrifuged at 700g for 5min before the cDNA was ethanol precipitated. As two LD-PCR reactions were carried out for each sample the purified samples were combined in an microfuge tube and 1/10th combined volume 3M sodium acetate was added along with 2.5 times the combined volume of ice cold ethanol. The microfuge tube was placed at -20°C for an 1h before being centrifuged at 13000g for 20min. The pellet was air dried for 10min before being resuspended in 20µl deionized water. The protocol says to discard the supernatant but in case cDNA has not properly precipitated it should be kept. The concentration and absorbance of the cDNA was checked using a Nanodrop (Agilent), if between 2-5µg cDNA have been obtained a library scale transformation can be performed. Further information about this procedure can be found in the Mate & Plate Library system (Clontech) manual (see Appendix 5.1).

2.6.5: cDNA Library Transformation

The cDNA library was co-transformed with pGADT7-Rec into S. cerevisae Y187. Due to the complementary ends of linearised pGADT7-Rec and the cDNA from the cDNA library, the vector and the cDNA fragments undergo in vivo recombination. The transformation was performed according to the library scale transformation protocol laid out in Yeastmaker 2 (Clontech) (see 2.5.6 and Appendix 5.1). The reaction mixture was as follows:

20µl ds cDNA from 2.5.4
6µl (0.5µg/µl) pGADT7-Rec
20µl 10µg/µl denatured Yeastmaker Carrier DNA
600µl competent cells
2.5ml PEG/LiAc

The reaction was incubated at 30°C for 45min with gentle mixing every 15min. 160µl

DMSO was mixed in and the cells were incubated in a 42°C water bath for 20min with gentle mixing every 5min. The reaction was centrifuged at 700g for 5min and the pellet was resuspended in 3ml YPD plus Medium. The transformation was incubated at 30°C with 40rpm shaking for 90min. The transformed cells were centrifuged at 700g for 5min before being resupended in 15ml 0.9% NaCl solution. The cells are now ready to be plated onto selective SD media.

Transformants were selected by plating onto SD/-Leu, as only cells containing the pGADT7-Rec vector are leucine autotrophs. We plated our transformants onto 24cm^2 plates containing SD/-Leu using the glass beads described in the Mate & Plate Library system manual (see Appendix 5.1). Two dilutions (10^{-1}, 10^{-2}) were also plated onto SD/-Leu plates and incubated for 3-4 days at 30°C. The transformants were incubated for 4 days at 30°C before being harvested, aliquoted and stored at -80°C. The transformants were harvested by scraping the colonies from the plates into 0.5l freezing media (150ml freezing media: 100ml YPDA, 50ml 75% glycerol). The cell density was estimated using a hemocytometer (cell density should not be $<2 \times 10^7$ or the suspension volume must be reduced by centrifugation) before being placed at -80°C.

2.7: Screening the cDNA Library

Before we carried out a yeast two hybrid screening we had first performed a control mating according to the Matchmaker Gold Yeast two hybrid system (Clontech) manual (see Appendix 5.1). Y2HG was transformed with pGBKT7-53 (a pGBKT7 vector encoding a murine p53), pGBKT7-Lam (pGBKT7 vector encoding lamin C). Y187 was transformed with pGADT7-T (pGADT7 vector encoding an SV40 large-T antigen). Transformations were performed according to the small scale mating procedure described in 2.4.6.

Single yeast colonies 2-3mm in size in the following combinations; Y2HG-pGBKT7-53 and Y187-pGADT7-T (positive control), Y2HG-pGBKT7-Lam and Y187-pGADT7-T (negative control) were suspended in 500µl 2X YPDA in a 1.5ml microfuge tube. These cultures were incubated at 30°C with shaking at 200rpm for 20-24h. 100µl of 1/10, 1/100 and 1/1000 dilutions were plated onto SD/-Trp, SD/-Leu, SD/-Trp/-Leu, SD/-Trp/-Leu/X/A media plates. After incubation at 30°C for 3-5 days; the positive control should have grown on all the plates, there should be a similar number of colonies

between SD/-Trp/-Leu and SD/-Trp/-Leu/X/A plates and the colonies on SD/-Trp/-Leu/X/A should be blue. The negative control should grow on all plates except SD/-Trp/-Leu/X/A.

We performed Yeast Two hybrid screenings according to the protocol laid out in the Matchmaker Gold Yeast Two Hybrid system (Clontech) (see Appendix 5.1), what follows is a brief description of those protocols. In order to determine whether the library aliquot has a high enough cfu/ml (>$2x10^7$), a number of dilutions were spread on SD/-Leu plates to determine the cfu/ml before the screening can begin. To perform a Yeast Two Hybrid screening, a 2-3mm yeast colony of the bait strain (Y2HG-pGBKT7-JAZ) was inoculated into 50ml of SD/-Trp media and incubated at 30°C until the OD_{600} reaches 0.8 (~16-20h). The culture was centrifuged at 1000g for 5min and the pellet was resuspended in SD/-Trp to a cell density >$1x10^8$. The bait strain was combined with a 1ml aliquot of the library strain (which had been thawed in a room temperature water bath) in a 2l flask with 45ml of 2xYPDA containing 50µg/ml kanamycin. The microfuge tube the library strain was stored in was washed twice with 1ml 2xYPDA and added to the 2l flask. The culture was incubated at 30°C for 20-24h with shaking at 40rpm.

After 20h the culture was checked for zygotes using a 40x phase contrast microscope, if no zygotes were present mating was allowed to continue for a further 4h. The cells were centrifuged at 1000g for 10min and the supernatant discarded, while the flask was rinsed twice with 50ml 0.5xYPDA (containing 50µg/ml kanamycin). The flask rinses were combined and were used to resuspend the pelleted cells which were centrifuged again at 1000g for 10min. The pelleted cells were resuspended in 10ml 0.5xYPDA (containing 50µg/ml kanamycin) and the volume of this final solution was measured.

100µl of 1/10, 1/100, 1/1000, 1/10000 dilutions was spread on the following plates; SD/-Trp, SD/-Leu, SD/-Trp/-Leu and incubated at 30°C for 3-5 days. The remainder of the culture was plated on 20 24cm^2 SD/-Trp/-Leu/X/A plates using sterile glass beads and were incubated at 30°C for 3-5 days. Any putative interactors that grew as blue colonies on the SD/-Leu/-Trp/X/A plates could be streaked out onto high stringency SD/-Ade/-His/-Leu/-Trp/X/A plates. Blue colonies that grew on the SD/-Ade/-His/-Leu/-Trp/X/A media underwent colony PCR using primers specific to pGADT7-Rec (see 2.8.2). Colonies with a pGADT7-Rec containing different sized inserts were sent off for DNA sequencing.

2.7.1: Independent Clones and Mating Efficiency

As part of the Library screening procedure it is important to calculate the number of screened clones and the overall mating efficiency.

The number of screened clones = colony forming units/ml of diploids x total resuspension volume

The resuspension volume refers to the total volume of cells and media when the diploids are resuspended in 10ml 0.5xYPDA.

Mating efficiency (%) = (cfu/ml of diploids / cfu/ml of limiting partner) x 100

The cfu/ml of limiting partner is calculated by comparing the cfu/ml of the SD/-Leu and SD/-Trp plates that the diploids were plated onto. The lowest cfu/ml is the limiting partner.

2.8: DNA Sequencing

2.8.1: Sequencing Baits

The primers in the Table 6 below were used for sequencing to check whether the inserts we had ligated into pGBKT7 were correctly in frame. The sequencing was done by Genome Enterprise (http://www.genome-enterprise.com/) who also supplied the forward primer T7.

Template Name	Forward Primer	Reverse Primer
pGBKT7-JAZ5	T7	GCAGGTCGACGGATCCCTATAGCCTTAGATCGAGATCTTTCG
pGBKT7-JAZ7	T7	CATGGAGGCCGAATTCATGATCATCATCATCAAAAACTGCGAC
pGBKT7-JAZ10.1	T7	GCAGGTCGACGGATCCTTAGGCCGATGTCGGATAGTAAG
pGBKT7-JAZ10.3	T7	GCAGGTCGACGGATCCTTACCTCTCCTTGCGCTTCTC
pGBKT7-JAZ4	T7	CTAATCTCTCCTTGCGCTTCTCGA

Table 6. Primer sequences of the reverse primers designed for use in sequencing pGBKT7-JAZ plasmids.

2.8.2: Sequencing Putative Interactors

The primers in the Table 7 below were designed to sequence the bait and prey plasmids of diploid yeasts that contained putative interactors. We also used these primers to check if our putative interactors were of different sizes (bp). These PCR reactions were run using Phusion Hot Start polymerase (NEB). We used a 30µl reaction volume, final concentrations of reagents were as follows;

1x Phusion Buffer (New England Bioscience) (from 5x)

200µM dNTPs

0.5µM primers (for both forward and reverse primers)

1pg-10ng DNA

0.02U/µl Phusion polymerase (New England Bioscience)

Reactions using these primers were run under the following conditions; 94°C 2min, 35 cycles of; 94°C for 30s, 55°C for 30s, 72°C for 1min. 72°C for 10min. We used the same method laid out in 2.4.7 to denature the yeast cells.

Primer Name	Sequence
pGBKT7 5’	GCGACATCATCATCGGAAGAGAG
pGBKT7 3’	CCTGACCTACAGGAAAGAGTTACTC
pGBKT7 3’ seq	CGGAATTAGCTTGGCTGCAAG
T7 - 2hybrid	TAATACGACTCACTATAGGGCG
pGADT7- Rec 3’ AD seq	CTGTGCATCGTGCACCATCT
pGADT7-Rec 5’ AD	CTATCTATTCGATGATGAAGATACCCCACC
pGADT7-Rec 3’ AD	AGTAT TACGATTCATCTGCAGCTC

Table. 7 Primers and their sequences that were designed for use in sequencing putative interactors found in yeast two hybrid assays.

3: Results

3.1: Yeast-two-hybrid – Bait Creation

To perform a Yeast two hybrid screen bait sequences were into the bait vector of the Matchmaker Gold Yeast-two-hybrid system; pGBKT7. It was shown that the sequences were cloned in frame and that the proteins were being correctly expressed. *JAZ*s were amplified from PD207 vectors using JAZ5, JAZ7 and JAZ10 specific primers (see 2.5.2). The primer combinations were as follows JAZ5: JAZ5FP/RP, JAZ7: JAZ7FP/RP, JAZ10.1: JAZ10FP/JAZ10.1RP,JAZ10.3:JAZ10FP/JAZ10.2/3RP, JAZ10.4:JAZ10FP /JAZ10.4RP. Resulting samples were run on a 1.2% agarose gel displayed in Fig. 13 below. The band sizes that were expected are as follows; JAZ5 - 825bp JAZ7 - 447bp JAZ10.1 - 594bp JAZ10.3 – 558bp. Figure 13 shows these expected bands, *JAZs* were amplified from PD207 twice, with subsequent gel electrophoresis giving the same result.

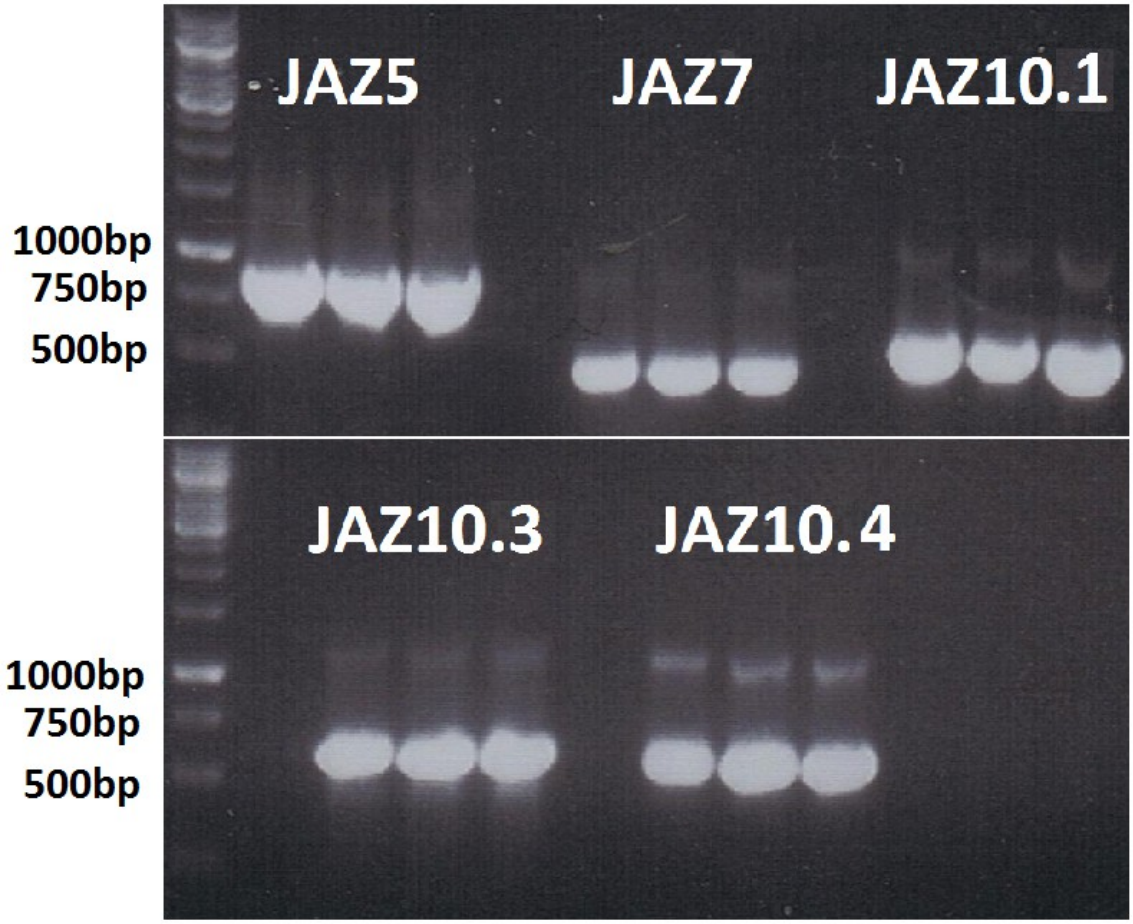


Fig. 13. Agarose gel electrophoresis of PCR performed using primers found in 2.4.2. The picture shows an ethidium bromide stained 1.2% agarose gel of PCR amplicons of PD207 vectors containing *JAZ5*, *JAZ7* and *JAZ10* sequences.

After successful amplification of JAZ sequences from the PD207 vectors, pGBKT7 was linearised using BamHI and EcoRI. In Fig. 14 there is a small amount of uncut pGBKT7 in the EcoR1 BamH1 lane but it was determined to be negligible. This enzyme digestion was performed twice with similar results. To create JAZ sequences with ends complementary to those in the linearised pGBKT7 vector, PCR was performed on the PD207-JAZ vectors with primers containing a 15bp homology to linearised pGBKT7 (see 2.5.3). As seen in Fig.14 similar sized bands to those in Fig. 13 were amplified and recovered using a Qiaquick gel extraction kit. The recovered product from the gel extraction was checked using gel electrophoresis (see Fig. 15). The gel extraction recovered JAZ sequences were ligated into the pGBKT7 vector linearised with BamH1-EcoR1 (see Fig. 14) using the In Fusion HD cloning system from Clontech. Once the fusion PCR was complete the vectors were transformed into *E. coli* and colony PCR was performed on the transformed *E. coli* to confirm the presence of the pGBKT7-JAZ constructs (see Fig. 16). As expected similar sized bands to those in Fig. 13 were visualized on the gel. A single transformation was performed as the results were confirmed by gel electrophoresis of the PCR product (see Fig. 16), enzyme digestion (see Fig. 17) and direct sequencing (see Appendix 5.2)

Colony PCR confirmed the presence of our JAZ sequences in the pGBKT7 vectors so restriction analysis was performed to check the sequences were cloned into the vectors correctly. Restriction enzymes were chosen where the pGBKT7-JAZ constructs contained restriction sites both inside and outside the JAZ sequence ligated into the vector. The results of the enzyme digestion were analysed on a 1.8% agarose gel and visualized using ethidium bromide (Fig. 17). The enzyme digestion was performed once as after PCR validation of the inserts, the recombinant pGBKT7 plasmids were sent to be sequenced by Genome Enterprise (the alignment data can be found in the Appendix 5.2). The sequencing confirmed the presence of JAZ sequences in the pGBKT7 vector.

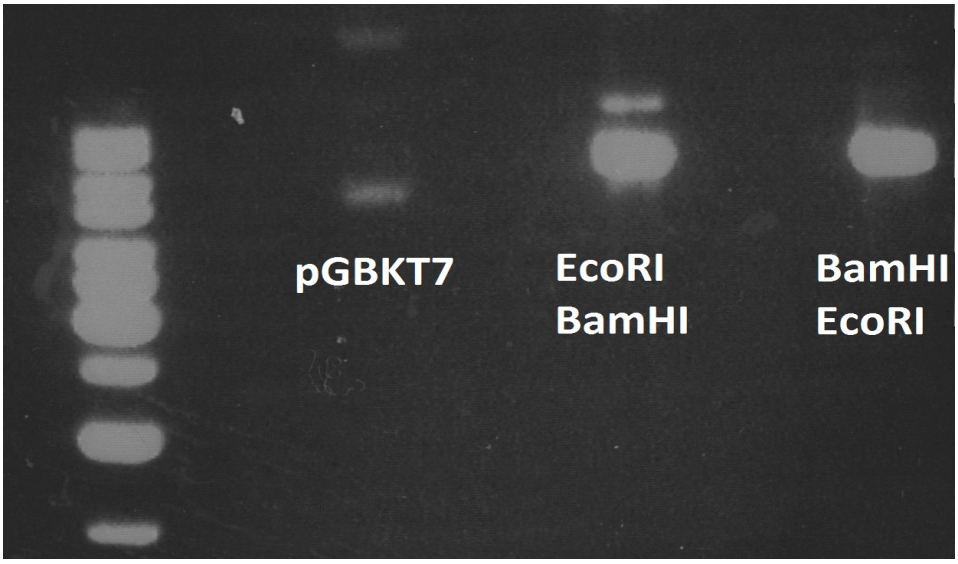


Fig. 14 Agarose gel electrophoresis of 3µl uncut pGBKT and 3µl pGBKT7 digested with 10U EcoRI and 10U BamHI for 1h. Two pGBKT7 digests were run because one sample was first digested with EcoRI then BamHI whereas the other was first digested with BamHI then EcoRI. In the picture above the former is first from the right of the sample labelled pGBKT7 while the latter is second from the right. The samples were run against 5µl (0.5µg/µl) Gene Ruler 1kb DNA ladder on a 1.2% agarose gel visualized with ethidium bromide.

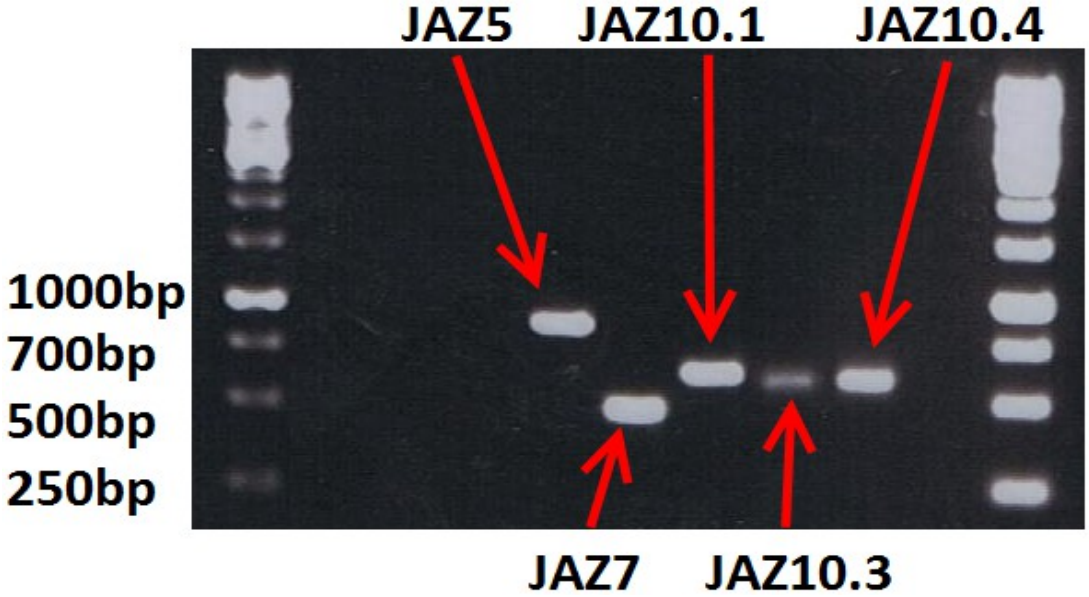


Fig. 15 Agarose gel electrophoresis of *JAZ5*, *JAZ7*, *JAZ10.1*, *JAZ10.3* and *JAZ10.4* amplified with primers as described in 2.5.3. 1µl of each gel extracted *JAZ* sequence was mixed with 5µl H_2O and 1µl loading buffer, each 7µl mixture was run against 2.5µl (left) and 5µl (right) Gene Ruler 1kb DNA ladder (0.5µg/µl) on a 1.2% agarose gel. The gel was visualized with ethidium bromide.

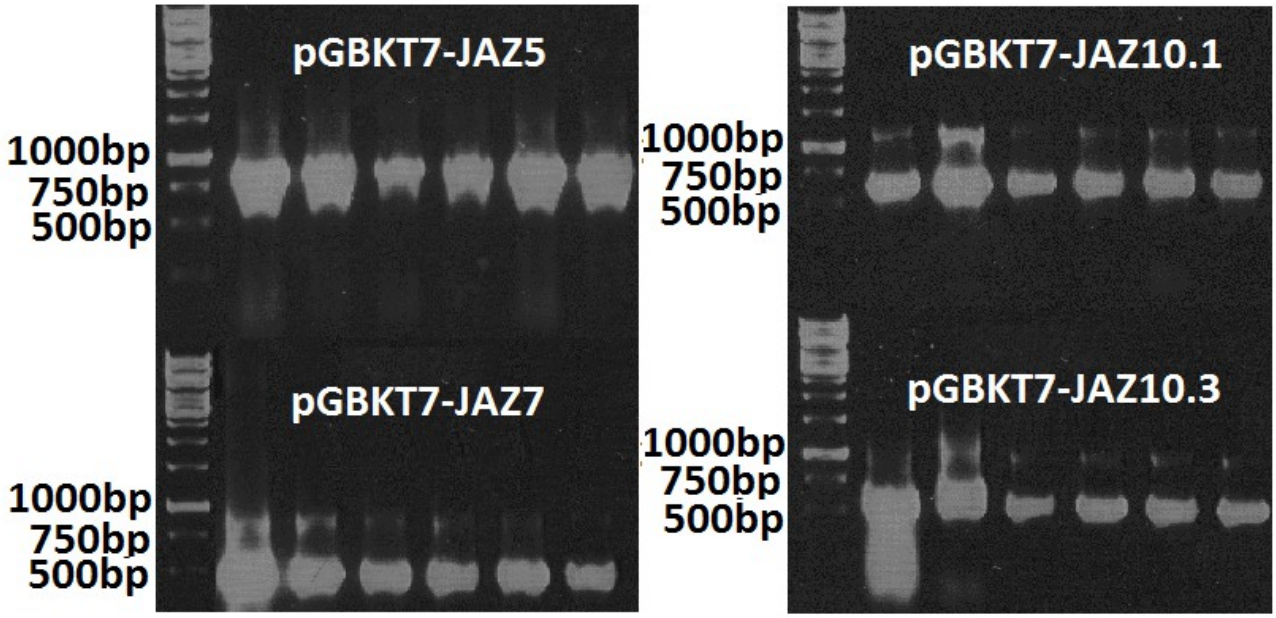


Fig. 16 Agarose gel electrophoresis of colony PCR from *E. coli* transformed with the pGBKT7-JAZ vectors from the In Fusion HD reaction. The colony PCR was performed on 6 individual colonies using primers described in 2.5.3. 7µl of each colony PCR reaction was run against 5µl Gene Ruler 1kb DNA ladder (0.5µg/µl) on a 1.2% agarose gel. The gel was visualized with ethidium bromide.

The pGBKT7-JAZ constructs were transformed into *Saccharomyces cerevisiae* Y2HGold using the protocol found in the Yeastmaker 2 manual (see 2.5.6). The Y2HGold transformants were plated onto SD/-Trp, as the pGBKT7 plasmid confers Trp autotrophy, and colony PCR was performed using the primers found in 2.5.3. The results of this colony PCR are found below in Fig. 18 where similar sized bands to those in Fig. 13 were amplified. This colony PCR experiment was performed twice, the repeat was performed to check Y2HGold transformants had retained the pGBKT7-JAZ constructs three months after the experiment shown in Fig. 18. The results of the later experiment are not shown as similar sized bands to those in Fig. 13 and Fig. 18 were amplified. A Western blotting was performed to confirm that the *JAZ*s genes were being correctly expressed. The protein extraction was first probed with a mouse anti-MYC antibody and then a secondary goat anti-mouse HRP conjugated antibody was used to bind the anti-MYC antibodies. The goat anti-mouse antibodies were visualized using chemiluminescence before being captured on photosensitive film (see Fig. 19). The bands that were visualized corresponded to the sizes that were expected: pGBKT7-53 – 57kDa, pGBKT7-JAZ5 – 52kDa, pGBKT7-JAZ7 – 38.9kDa pGBKT7-JAZ10.1 – 43.8kDa pGBKT7-JAZ10.3 – 42.5kDa. The Western blotting was successfully repeated

twice with similar results. A coomasie stained blot of the same protein extraction used in the Western blotting shown in Fig. 19, is included in the Appendix (see 5.3).

As well as controlling that the transformed Y2HG were correctly expressing the JAZ proteins, the reporter genes were checked to ensure they were not being expressed autonomously. All the Y2HG containing pGBKT7-JAZ constructs were found to be unable to grow on media containing Aurabasidin A while diploid yeast created during a control mating (see 2.7) were able to grow. The JAZ proteins were established to not be toxic when expressed in Y2HG by comparing growth of Y2HG transformed with empty pGBKT7 vector with Y2HG transformed with pGBKT7-JAZ vectors. There was no significant difference (these results are not shown).

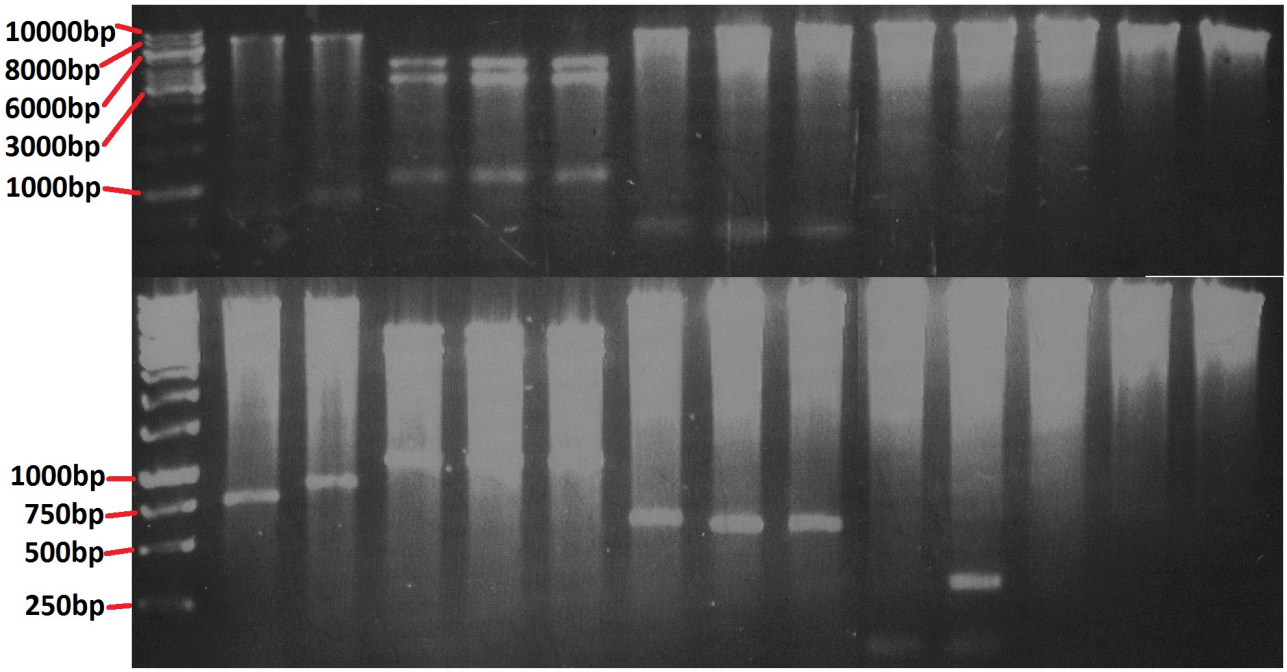


Fig. 17 Enzyme digestion carried out on pGBKT7-JAZ vectors. The lanes from left to right contain; JAZ5 Nco1, JAZ5 BamH1/EcoR1, JAZ10.1 Xba1, JAZ10.3 Xba1, JAZ10.4 Xba1, JAZ10.1 BamH1/EcoR1, JAZ10.3 BamH1/EcoR1, JAZ10.4 BamH1/EcoR1, JAZ7 BamH1, JAZ7 BamH1/EcoR1, JAZ10.1EcoR1/Cla1, JAZ10.3 EcoR1/Cla1, JAZ10.4 EcoR1/Cla1. 10µl of each digestion was loaded. JAZ7 BamHI, JAZ7 BamHI/EcoRI, JAZ10.1 EcoRI/ClaI, JAZ10.3 EcoRI/ClaI and JAZ10.4 EcoRI/ClaI digestions contained small fragments (<500bp) so 17µl was loaded. The image is a composite of two images of the same 1.2% agarose gel visualized with ethidium bromide using different settings to highlight the larger bands (top image) and the smaller bands (bottom image).

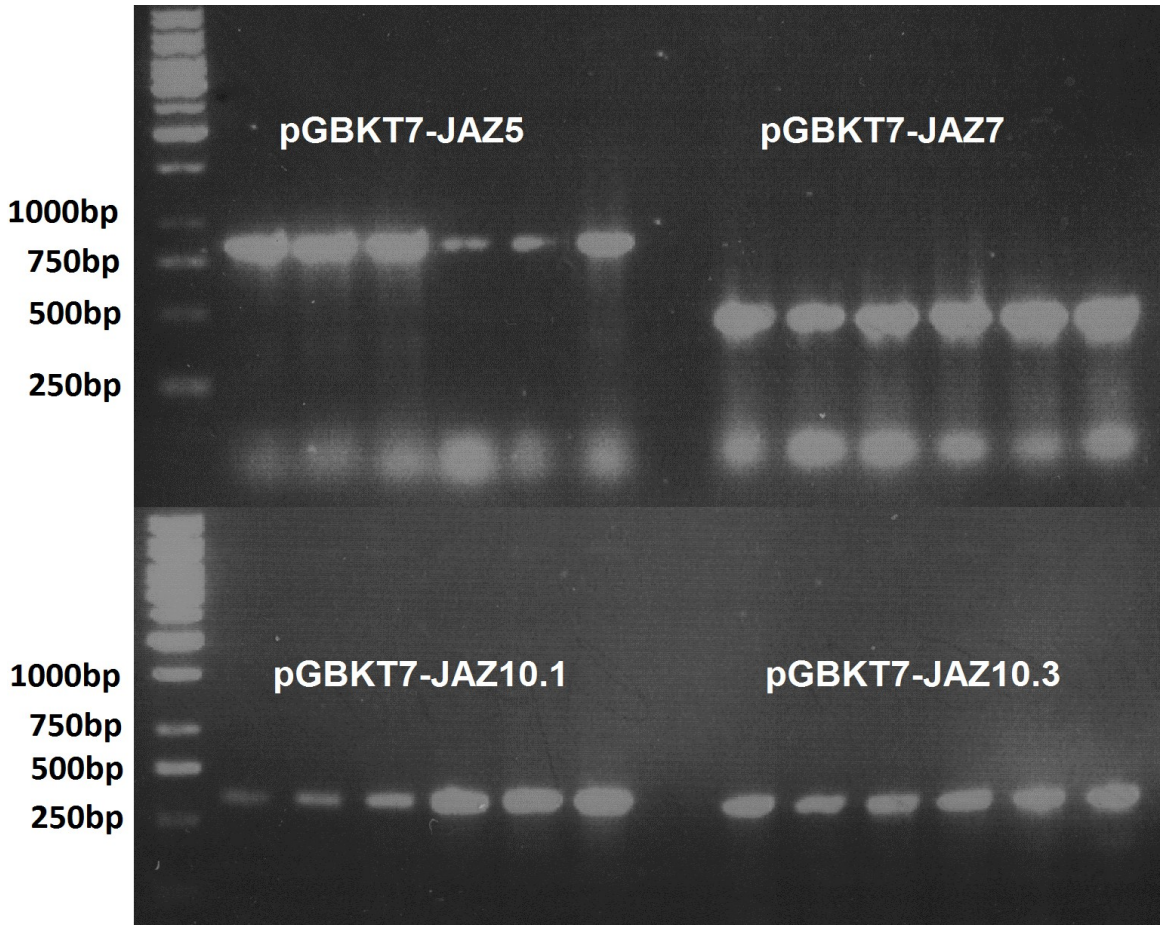


Fig. 18. Agarose gel electrophoresis of colony PCR from Y2HGold transformed with pGBKT7-JAZ constructs. Colony PCR was performed on 6 randomly selected colonies of Y2HGold transformed with pGBKT7-JAZ constructs using primers found in 2.5.3. 5µl of each reaction was run alongside 7µl Gene Ruler 1kb DNA ladder (0.5µg/µl) on a 1.2% agarose gel. The gel was visualized with ethidium bromide.

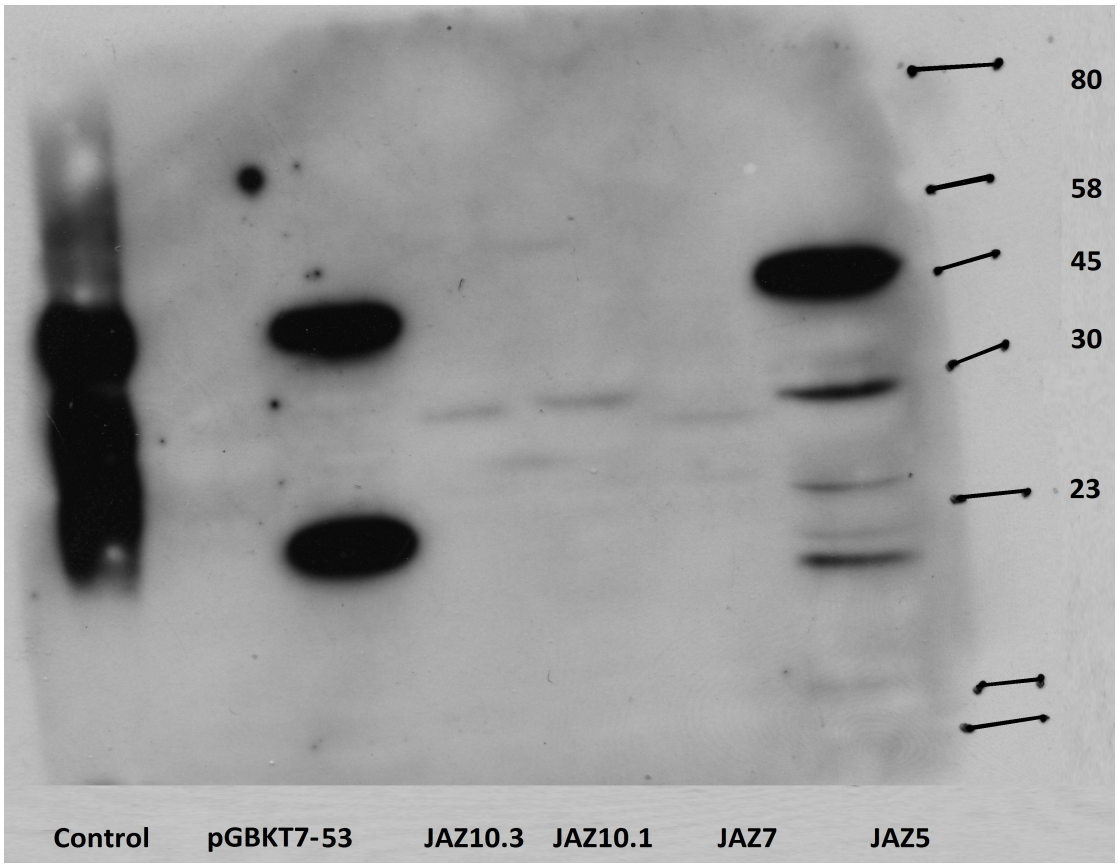


Fig. 19. Western blot of protein extractions taken from Y2HGold cells transformed with pGBKT7-JAZ5, pGBKT7-JAZ7, pGBKT7-JAZ10.1, pGBKT7-JAZ10.3 and pGBKT7. The control is Positope control protein. The protein ladder used as a size marker is labelled in kDa.

3.2: Yeast-two-hybrid – Prey library construction

In order to create a cDNA library to screen against, cDNA was created from mRNA species, this cDNA was then ligated into the prey vector (pGADT7-Rec) whereafter the pGADT7-cDNA library was transformed into *S. cerevisae* Y187 (Y187). After creation of the RNA species (see 2.6.1) a Nanodrop (Agilent) was used to test the concentration of RNA in the samples and to check for possible contaminants. These results are presented in Table 8 below, the results are well within acceptable ranges and concentrations. The quality of the RNA was verified using gel electrophoresis (Fig. 20). Upon confirming that the RNA species were of suitable quality, creation of cDNA libraries for use with the Matchmaker Gold yeast-two-hybrid system began. Due to the amount of RNA available, only a single cDNA library could be created per RNA

species. First strand synthesis (see 2.6.2) was performed after which LD-PCR (see 2.6.3) was used, 7µl of each sample obtained through LD-PCR was analysed by gel electrophoresis (see Fig. 21). The results obtained from the gel electrophoresis were comparable to those displayed in the Mate and Plate user manual (see Appendix 5.1). Following validation of the LD-PCR reaction each reaction was purified in a Chroma Spin TE-400 column (see 2.6.4). The resulting purified double stranded cDNA was analysed using a Nanodrop (Agilent) (see Table 9 below). Once the cDNA libraries concentrations had been tested the two cDNA libraries were co-transformed with the recombinant vector pGADT7-Rec into Y187. Transformation of Y187 using the DC3000 library was performed twice. The number of independent clones produced by the transformation was calculated to ensure that the libraries were of a suitable complexity, these results are displayed in Table 10.

RNA species	RNA concentration (ng/µl)	Absorbance (260nm/280nm)	Absorbance (260nm/230nm)
Mixed	671.3	2.09	2.02
DC3000	541.7	2.13	2.05
hrpA	452.6	2.05	2.07

Table 8. RNA concentration and absorbance ratios of the RNA species used to create the cDNA libraries.

cDNA library	DNA concentration (ng/µl)	Absorbance (260nm/280nm)	Absorbance (230nm/260nm)
Mixed	238.6	1.6	2.36
DC3000	261.1	1.56	2.49
hrpA	105.9	1.68	2.39

Table 9. DNA concentrations and absorbance ratios of double stranded cDNA libraries after purification and precipitation as measured by Nanodrop (Agilent).

Library Y187 co-transformed	Independent clones
DC30001	585000-930000
DC30002	23000
Mixed	405000-770000
hrpA	990000

Table 10. Numbers of independent clones produced from each transformation. DC30001 refers to a Y187 transformation performed 29/3/12, DC30002 was a second transformation performed 3/7/12.

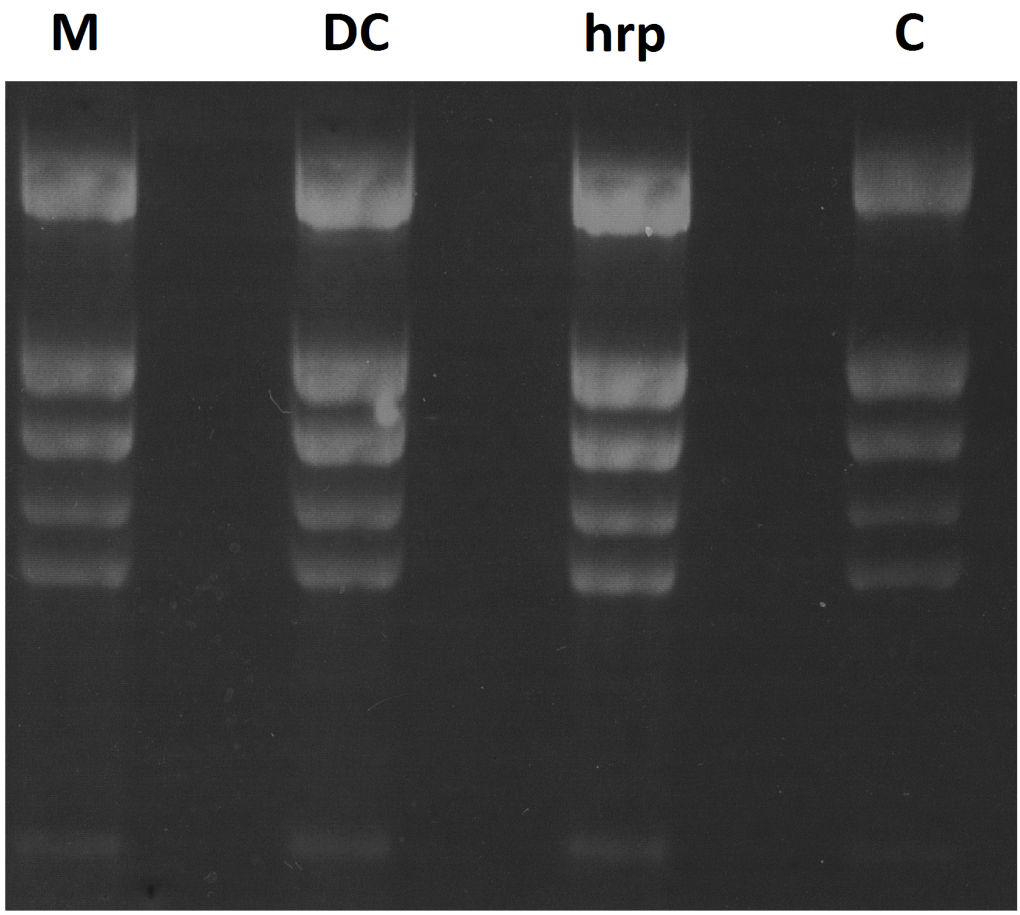


Fig. 20 RNA gel electrophoresis of RNA species. M is the Mixed RNA species, DC is the DC3000 infected leaf RNA species and hrp is the HrpA infected leaf RNA species. C is a control RNA sample of Mouse poly A+ RNA supplied with the Mate & Plate library system. 2µg of each RNA species was run on a 1.5% denaturing agarose gel. The gel was visualized using ethidium bromide.

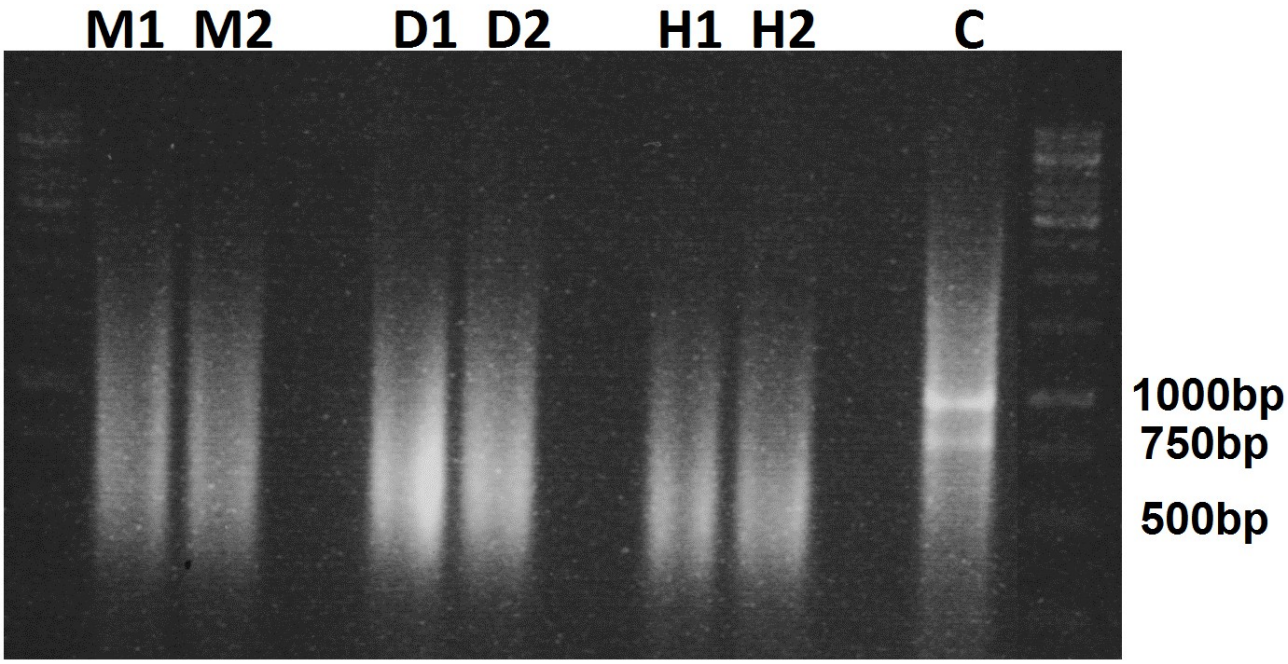
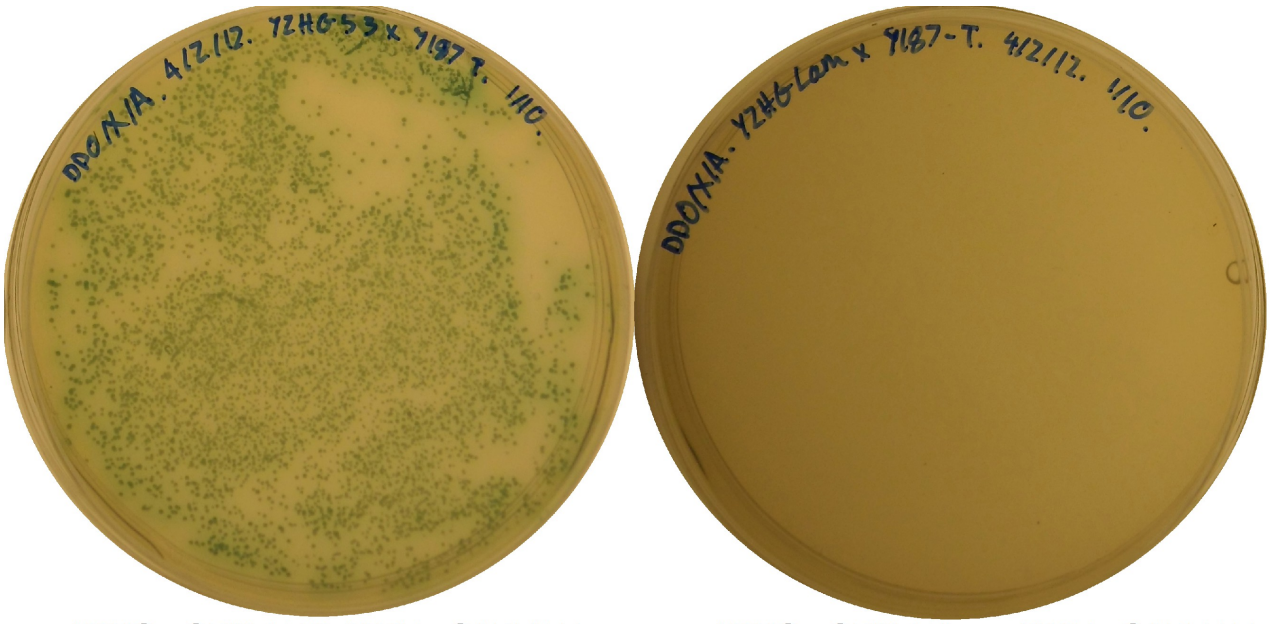


Fig. 21 Agarose gel electrophoresis of LD-PCR samples. Two LD-PCR reactions are performed per sample; M1 and M2 are Mixed RNA species reactions, DC1 and DC2 are DC3000 RNA species reactions and H1 and H2 are HrpA library reactions. C is Mouse Liver Poly A+ RNA used as a control. 7µl of each LD-PCR reaction was run alongside 0.25µl and 0.5µl 1kb GeneRuler DNA Ladder (0.5µg/µl) loaded on the left and right side of the gel. The gel was visualized with ethidium bromide.

3.3: Yeast-two-hybrid – Screening

To perform a yeast two hybrid screening the correct mating of yeast needed to be verified. In order to do thisY2HG was transformed with pGBKT7-53 and pGBKT7-Lam, while Y187 was transformed with pGADT7-T. In a mating between Y2HG-pGBKT7-53 and Y187-pGADT7-T diploids are formed that are able to grow on SD/-Trp/-Leu/X/A while diploids formed by mating Y2HG-pGBKT7-Lam with Y187-pGADT7-T cannot. Additionally a similar number of Y2HG-pGBKT7-53xY187-pGADT7-T colonies should be present on SD/-Trp/-Leu and SD/-Trp/-Leu/ containing Aurabasidin A. The results of the control mating are shown below in Fig. 22 where it can be seen that the control mating behaved as expected. This control experiment was also performed alongside the yeast two hybrid screenings to confirm that growth media was correctly selective.



Y2HG-pGBKT7-53xY187-pGADT7-T **Y2HG-pGBKT7-LamxY187-pGADT7-T**

Fig. 22. Control mating Y2HG-pGBKT7-53 and Y2HG-pGBKT7-Lam with Y187-pGADT7-T. 100µl of a 1/10 dilution of Y2HG-pGBKT7-53xY187-pGADT7-T and Y2HG-pGBKT7-LamxY187-pGADT7-T were plated on SD/-Trp/-Leu/X/A plates. Only colonies that can activate *AUR1-C* can form colonies on plates containing AbA, activation of *MEL1* causes blue colonies to form (see 2.5). As only Y2HG-pGBKT7-53xY187-pGADT7-T can activate GAL4 transcription it can form colonies on DDO/X/A media.

After control validation experiments two yeast two hybrid screenings were performed using Y2HG-pGBKT7-JAZ5 as bait and screening it against the DC3000 library. Following screenings the number of screened clones and the overall mating efficiency (see Table 11) was calculated. Seven putative interactors from the two screenings were obtained, using PCR it was verified that the colonies contained pGADT7-Rec containing a cDNA insert before being sent for sequencing. The colonies were streaked on SD/-Trp/-Leu/-His/-Ade/X/A plates and the surviving colonies underwent PCR. PCR was performed using the primers found in 2.8.2. Gel electrophoresis using samples of three of these reactions visualized with ethidium bromide are displayed below in Fig. 23 and Fig. 24. As the colony PCR in Fig. 23 and Fig. 24 showed that pGADT7-Rec was present in all of the colonies tested, a Qiagen PCR purification kit was used to purify the PCR reactions. The purified DNA was then sent to be sequenced. The full sequences for these interactors can be found in the Appendix (see 5.2). BLASTN 2.2.27 was used to

analyse the sequencing data, the results of which are displayed in Table 12 below.[164]

Library Screening	Independent clones screened	Mating Efficiency
JAZ5 I	3.16×10^7	17.53%
JAZ5 II	1.9×10^8	11.5%

Table 11. The number of independent clones screened in our yeast two hybrid screenings and the mating efficiency of Y2HG-pGBKT7-JAZ5 and Y187-pGADT7-Rec. JAZ5 I refers to the first library screening, JAZ5 II refers to the second library screening performed.

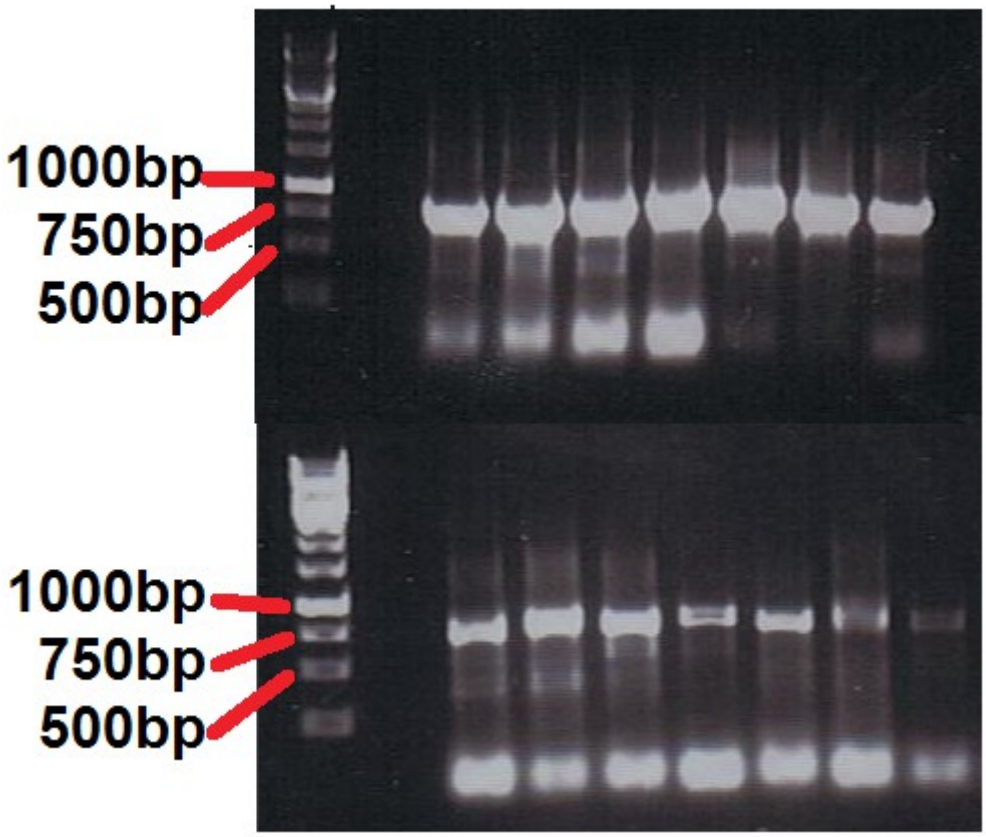


Fig. 23 Agarose gel electrophoresis of a colony PCR using putative interactors of a yeast two hybrid screen. The top gel shows samples from colony PCR run using T7 and pGADT7-Rec 3' AD primers, the bottom gel shows samples from a PCR using T7 and pGADT7-Rec 3' AD seq primers (see 2.8.2). 5µl of each colony PCR reaction was run alongside 7µl of 1kb GeneRuler DNA Ladder (0.5µg/µl). The 1.2% agarose gel was visualized using ethidium bromide.

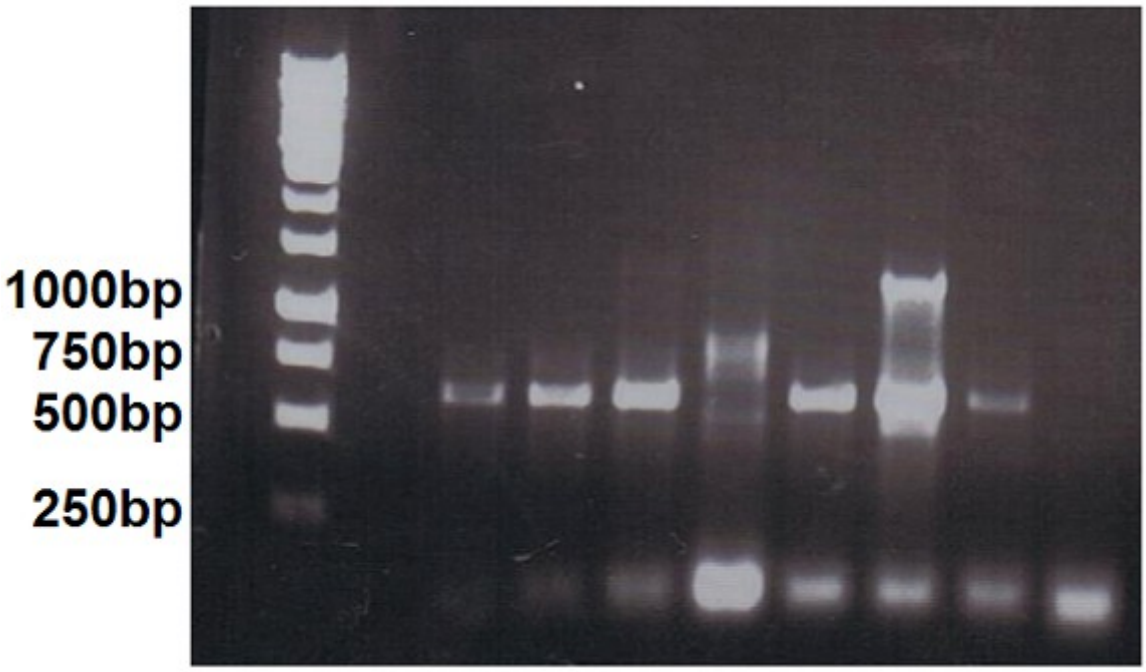


Fig. 24 Agarose gel electrophoresis of a colony PCR using putative interactors of a yeast two hybrid screen. The gel shows samples of a PCR run using pGADT7-Rec 5' AD and pGADT7-Rec 3' AD seq primers (see 2.8.2). 5µl of each colony PCR reaction was run alongside 7µl of 1kb GeneRuler DNA Ladder (0.5µg/µl). The 1.2% agarose gel was visualized using ethidium bromide.

Colony Number	Accession	Query Coverage	Maximum identity	E value	Feature Name
1	NC_003076.8	71%	552/555	0.0	HSC70-1
2	NC_003076.8	72%	552/555	0.0	HSC70-1
3	NC_003076.8	71%	552/555	0.0	HSC70-1
4	NC_003071.7	73%	595/593	0.0	GSTF10
5	NC_003076.8	71%	552/555	0.0	HSC70-1
6	NC_003076.8	72%	552/555	0.0	HSC70-1
7	NC_003076.8	72%	552/555	0.0	HSC70-1

Table 12. Results of BLASTN alignment on the sequences received from sequencing diploid colonies recovered from a yeast two hybrid screening. These results are compiled from output from http://blast.ncbi.nlm.nih.gov/. The sequences used for this alignment were sequenced by Genome Enterprise using the T7 primer (see 2.8.2)

3.4: JAZ mutant susceptibility to *Botrytis cinerea*

At-jaz mutants were tested for increased susceptibility to *B. cinerea*. As *B. cinerea* activates the SA signalling pathway to antagonize JA/Et mediated fungal defences it was hypothesized that *At-jaz* mutants could show different susceptibilities to necrotrophic pathogen. A photograph of the *B. cinerea* phenotypes of *At-jaz* mutants is presented in Fig. 27. The size of the lesions was determined using ImageJ and the average lesion size is shown below in Fig. 25 and Fig. 26. The experiment was performed three times, although the results of the third experiment are not shown here. As there were 8 Col-0 leaves per plate and only 4 leaves per *At-jaz* mutant, the leaves from two plates (see Fig. 27) were combined and the lesion size on the leaves was analysed. Fig. 25 and Fig. 26 show the average lesions sizes of *At-jaz* leaves from two plates but average Col-0 lesion size from the individual plates. Fig. 25 and Fig. 26 show that of all the mutants *At-jaz5/7* and *At-jaz7/10* displayed a significant increase in the average lesion size, this can also be seen in Fig. 27. It is curious that the *At-jaz5/7/10* mutant does not display significantly increased lesion sizes, as both *At-jaz5/7* and *At-jaz7/10* mutants do display significantly increased lesion sizes compared to Col-0. However both Fig. 25 and Fig. 26 show that there is no significant increase in the lesion sizes on *At-jaz5/7/10* compared to Col-0.

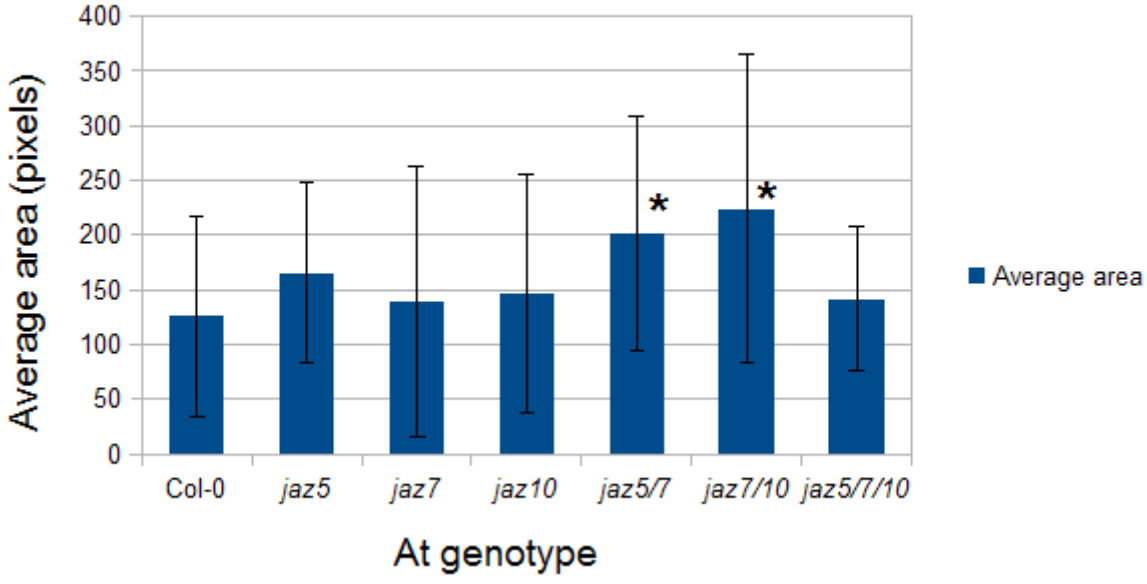


Fig. 25 Average lesion size present on 8 leaves of various *At-jaz* mutants and Col-0 3dpi with *B. cinerea*. Lesion size was calculated using ImageJ, error bars denote the standard deviation and asterisks denote significant differences ($p<0.05$) in average lesion area

compared to Col-0. The Col-0 leaves that had their lesion size analysed for this graph are at the top of Fig. 27.

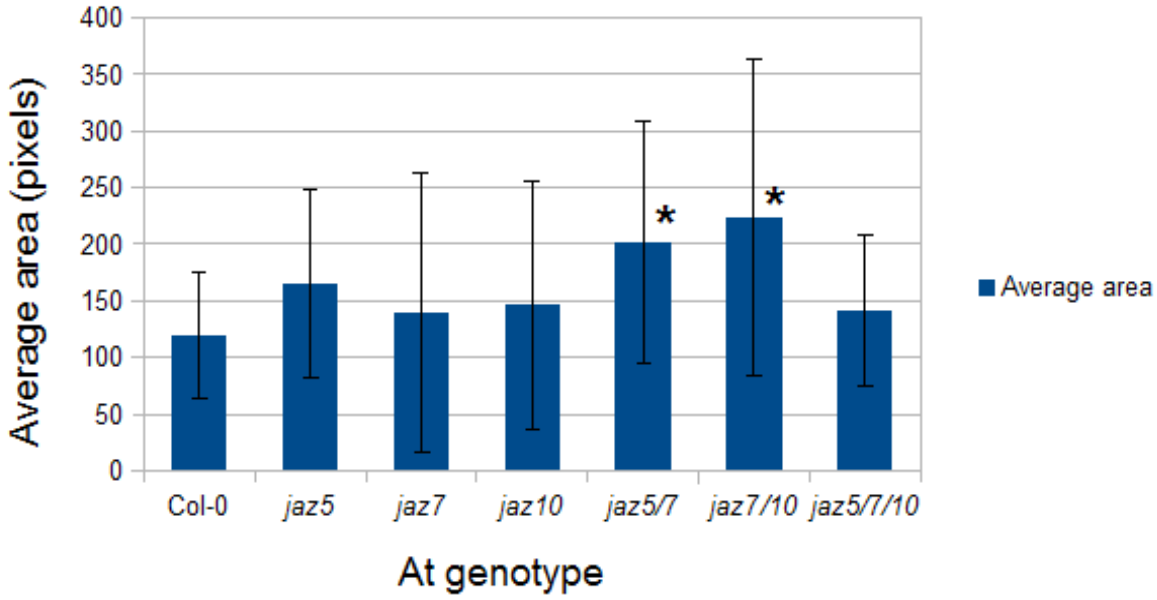
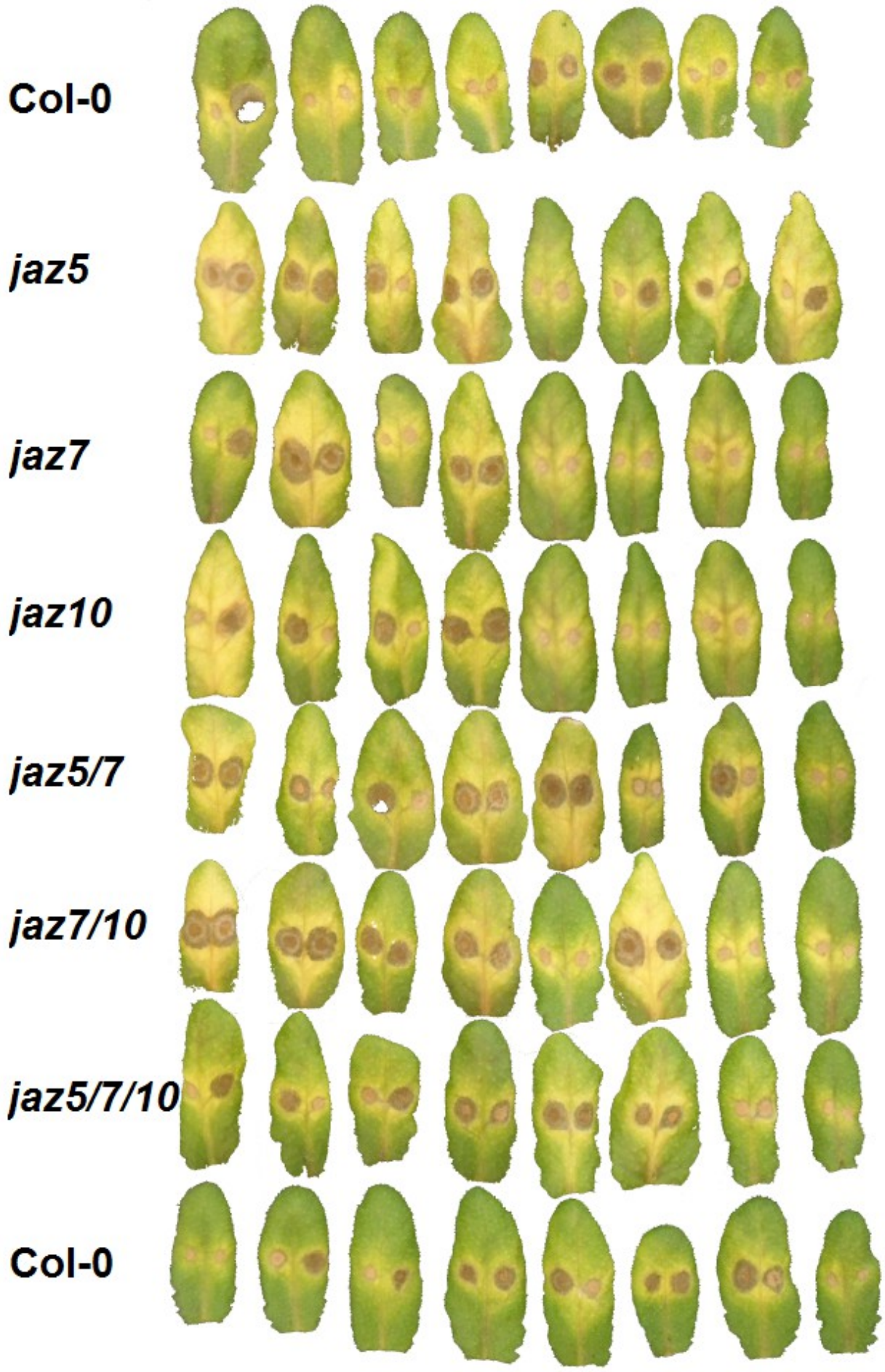


Fig. 26 Average lesion size present on 8 leaves of various *At-jaz* mutants and Col-0 3dpi with *B. cinerea*. Lesion size was calculated using ImageJ, error bars denote the standard deviation and asterisks denote significant differences ($p<0.05$) in average lesion area compared to Col-0. The Col-0 leaves that had their lesion size analysed for this graph are at the bottom of Fig. 27

Fig. 27 (Page below) Phenotypes on *At-jaz* mutants and Col-0 3dpi (days post infection) with *B. cinerea* spores. The *At-jaz* mutants are arranged such that the first four leaves from the left were from the same plate as the Col-0 at the top of the picture, the *At-jaz* mutants on the right are from the same plate as the Col-0 at the bottom of the picture.


Col-0
jaz5
jaz7
jaz10
jaz5/7
jaz7/10
jaz5/7/10
Col-0

3.5: Amino Acid Transport Mutants

We have previously shown that there is a significant difference in GABA, proline and glutamate levels between Col-0 and *At-at5g41800/gat-1* plants infected with PtsD *hrpA-* so we performed further experiments to extend our knowledge (see Fig. 28 and 29). *At* was infiltrated with PtsD and samples were harvested at 8, 16 and 18hpi while samples from uninfected plants were used to establish a base line for the amino acids. This experiment was performed three times, but the samples for the third experiment were lost due to equipment malfunction. Col-0 and *At-at5g41800/gat-1* plants were also infiltrated with PtsD *hrpA-* (see Fig. 30) as a large difference in amino acid levels between Col-0 and *At-at5g41800/gat-*1 treated with PtsD and PtsD *hrpA-* had previously been observed. This experiment was only performed once due to time constraints. The effect of GABA on PstD growth in amino acid transporter mutants; *At-gat-1*, *At-at5g41800*, *At-at5g41800/gat-1* was investigated. It has been reported that GABA could repress HR in *N. tabacum* infiltrated with PtsD and that GABA could potentially inhibit expression of hrp locus genes.[131] PstD and different GABA concentrations were inoculated into *At* mutants and the cfu/ml (see 2.2.1) were measured. Fig. 31 and Fig. 32 display the results for two of these experiments. The experiment was performed three times.

Figure 28 shows significant lower relative responses for GABA in *At-gat-1* and *At-at5g41800* compared to Col-0 at all tested time points. There is also a significant difference in basal GABA levels in *At-at5g41800/gat-1* compared to Col-0 but no significant differences are present at later time points. There are some significant differences in GABA levels between the mutants, *At-gat-1* shows significant differences to *At-at5g41800/gat-1* at all time points. *At-gat-1* and *At-at5g41800* show significant differences in basal GABA levels and at 16hpi. *At-at5g41800* and *At-at5g41800/gat-1* have similar basal GABA levels but 8 and 16hpi *At-at5g41800/gat-1* has significantly higher GABA levels. The relative responses for proline are significantly lower compared to Col-0 at all time points for *At-gat-1* and *At-at5g41800. At-at5g41800/gat-1* shows a significantly higher response for proline compared to Col-0 at 16hpi. *At-at5g41800/gat-1* also has significantly higher responses for proline compared to the other mutants at all time points. The relative responses for proline of *At-gat-1* and *At-at5g41800* are significantly different at 8hpi. Glutamate responses were quite different as only *At-gat-1* showed a significantly lower response during all time points compared

to Col-0 and *At-at5g41800/gat-1* at all time points. *At-at5g41800* showed no significant differences compared to Col-0, although it was significantly different to *At-gat-1* at 0h and *At-at5g41800/gat-1* at 16hpi. *At-at5g41800/gat-1* showed significant differences to Col-0 at 0h and 16hpi.

An independent experiment (Fig. 29) shows results for the same amino acids but using a slightly longer maximum time point of 18hpi. *At-gat-1* displayed significantly lower relative responses for GABA compared to Col-0 at 0 and 18hpi. *At-gat-1* also gave significantly lower relative responses for GABA at 0h than the other mutants. At 8hpi the GABA relative response was significantly higher for *At-gat-1* than *At-at5g41800.* At 18hpi there were no significant differences in GABA between any of the mutants. *At-gat-1* shows a significantly lower relative response for proline at 8hpi compared to Col-0 while at 18hpi the relative response of *At-gat-1* is significantly higher. *At-gat-1's* basal relative response for proline is significantly higher than that of *At-at5g41800* but significantly lower than *At-at5g41800/gat-1.* At 8hpi *At-gat-1*'s response is significantly lower than that of *At-at5g41800/gat-1* and at 18hpi it is significantly higher than the responses of *At-at5g41800* and *At-prot-1*. *At-gat-1* shows no significant differences for relative responses for glutamate compared to Col-0 or any other mutant.*At-at5g41800* showed a significantly higher response for GABA than Col-0 or any other mutant at 0hpi and a significantly lowered response for GABA at 8 and 18hpi compared to Col-0. At 8hpi the response to GABA was also significantly lower than the other mutants, while at 18hpi there was no significant difference between any of the mutants. In comparison to all other genotypes the basal relative response for proline is significantly lower for *At-at5g41800.* At 8hpi *At-at5g41800* shows a significantly lower response than Col-0 and *At-at5g41800/gat-1* while at 18hpi the response is significantly lower than that of *At-gat-1* and *At-at5g41800/gat-1. At-at5g41800*'s 0h response to glutamate is significantly lower than that of *At-prot-1.* The other significant differences in relative response to glutamate occur at 18hpi where *At-at5g41800* is significantly lower than both *At-at5g41800/gat-1* and *At-prot-1.*

At-at5g41800/gat-1 responses to GABA were significantly lower than Col-0 at 18hpi. At 0hpi the response was significantly lower than the response of *At-at5g41800*, but significantly higher than the response of *At-gat-1.* At 8hpi the relative response of *At-at5g41800/gat-1* was significantly higher than that of *At-at5g41800. At-at5g41800/gat-1* gave the largest relative responses for proline, which were significantly higher than

those of Col-0 at 18hpi. Both at 0h and at 8hpi relative responses for proline were significantly increased for *At-at5g41800/gat-1* compared to *At-gat-1*, *At-at5g41800* and *At-prot-1*. At 18hpi *At-at5g41800/gat-1*'s response was significantly increased compared to *At-at5g41800* and *At-prot-1.* For glutamate the only significant difference is at 0h where *At-at5g41800/gat-1*'s relative response is significantly lower than that of *At-prot-1*.

At-prot-1 showed a significantly higher GABA response than *At-at5g41800/gat-1* and *At-at5g41800* at 0h, while at 8hpi the response was significantly higher than *At-at5g41800.* For proline *At-prot-1* shows significantly lower responses than Col-0 at 0h and 8hpi. Additionally *At-prot-1* has a significantly higher response than *At-at5g41800* but a significantly lower response than *At-at5g41800/gat-1* at 0h. At 8 and 18hpi the relative response of *At-prot-1* is significantly lower than that of *At-at5g41800/gat-1*. At 18hpi *At-gat-1* also displays a significantly higher relative response than *At-prot-1.*

Fig. 30 measures the same amino acids as Fig. 28 and Fig. 29 but Col-0 and *At-at5g41800/gat-1* were treated with PtsD *hrpA* instead of PtsD to see if changes in amino acid levels would be affected. The two significant differences found were for GABA at 8hpi and 16hpi in *At-at5g41800/gat-1,* at 8 hpi the double mutant had significantly higher relative response than Col-0 for GABA whereas at 16hpi the response was significantly lower. Fig. 31 shows significantly reduced PtsD growth for *At-at5g41800* treated with PtsD and 10mM GABA compared to identically treated Col-0. While *At-gat-1* shows no significant differences *At-at5g41800/gat-1* shows significant decreases in PtsD growth for all treatments compared to Col-0. Fig. 32 shows that *At-at5g41800, At-gat-1* and *At-at5g41800/gat-1* all have significantly increased growth compared to Col-0 for treatment with PtsD alone while only *At-at5g41800/gat-1* has significantly decreased growth for treatment with PtsD and 10mM GABA.

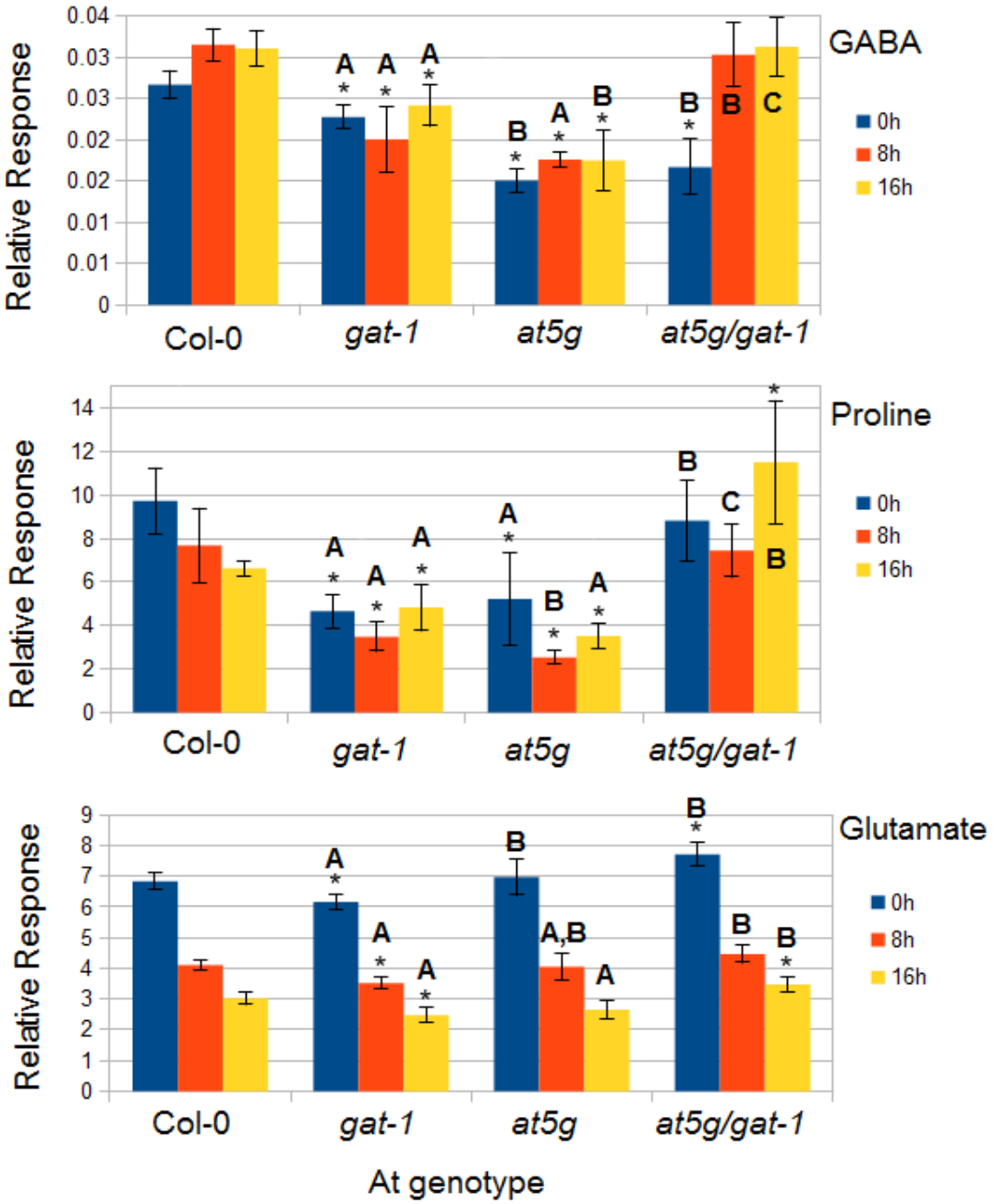


Fig. 28. Amino acid differences between GABA transporter mutants. The graphs shows relative responses for GABA, Proline and Glutamate in Col-0, *At-gat-1, At-at5g41800* (labelled at5g), *At-at5g41800/gat-1* (labelled at5g/gat-1) treated with PstD at 8 and 16hpi. The relative responses shown are the average of 3 samples each containing 3 leaves from two plants. The samples taken at 0h were taken from uninfected plants. The error bars indicate standard deviation, asterisks indicate significant differences in relative responses compared to Col-0 ($p<0.05$) calculated using Student's t-test. Different letters above bars indicate significant differences ($p<0.05$) in average relative responses between *At* mutants calculated using students t-test, if two letters are shown significant differences only exist between bars that do not share any letters.

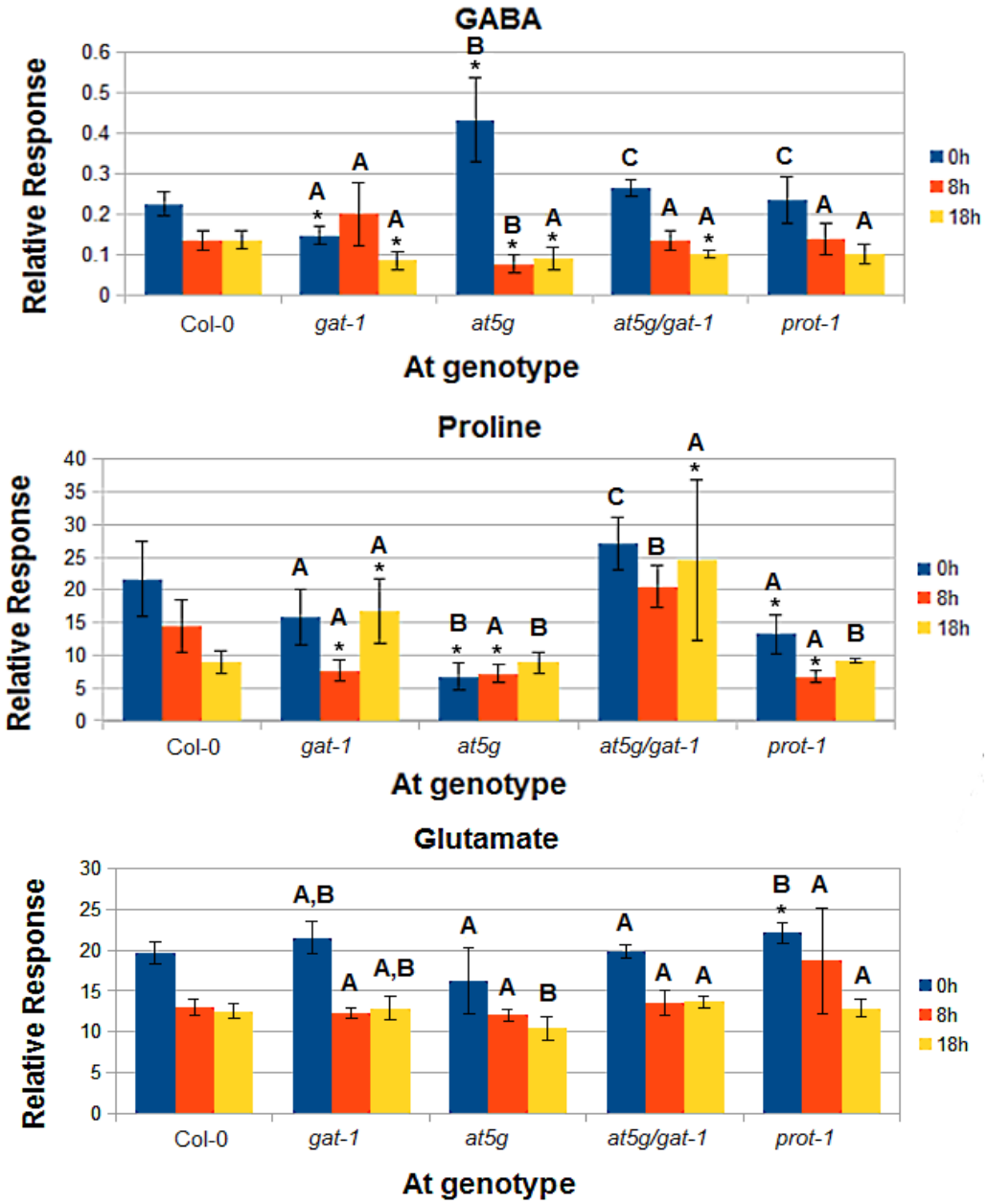


Fig. 29. Amino acid differences between GABA transporter mutants. The graphs shows relative responses for GABA, Proline and Glutamate in Col-0, *At-gat-1, At-at5g41800* (labelled at5g), *At-at5g41800/gat-1* (labelled at5g/gat-1) treated with PstD at 8 and 18hhpi. The relative responses shown are the average of 3 samples each containing 3 leaves from two plants. The samples taken at 0h were taken from uninfected plants. The error bars indicate standard deviation, asterisks indicate significant differences in relative responses compared to Col-0 ($p<0.05$) calculated using Student's t-test. Different letters above bars indicate significant differences ($p<0.05$) in average relative responses between *At* mutants calculated using students t-test, if two letters are shown significant differences only exist between bars that do not share any letters.

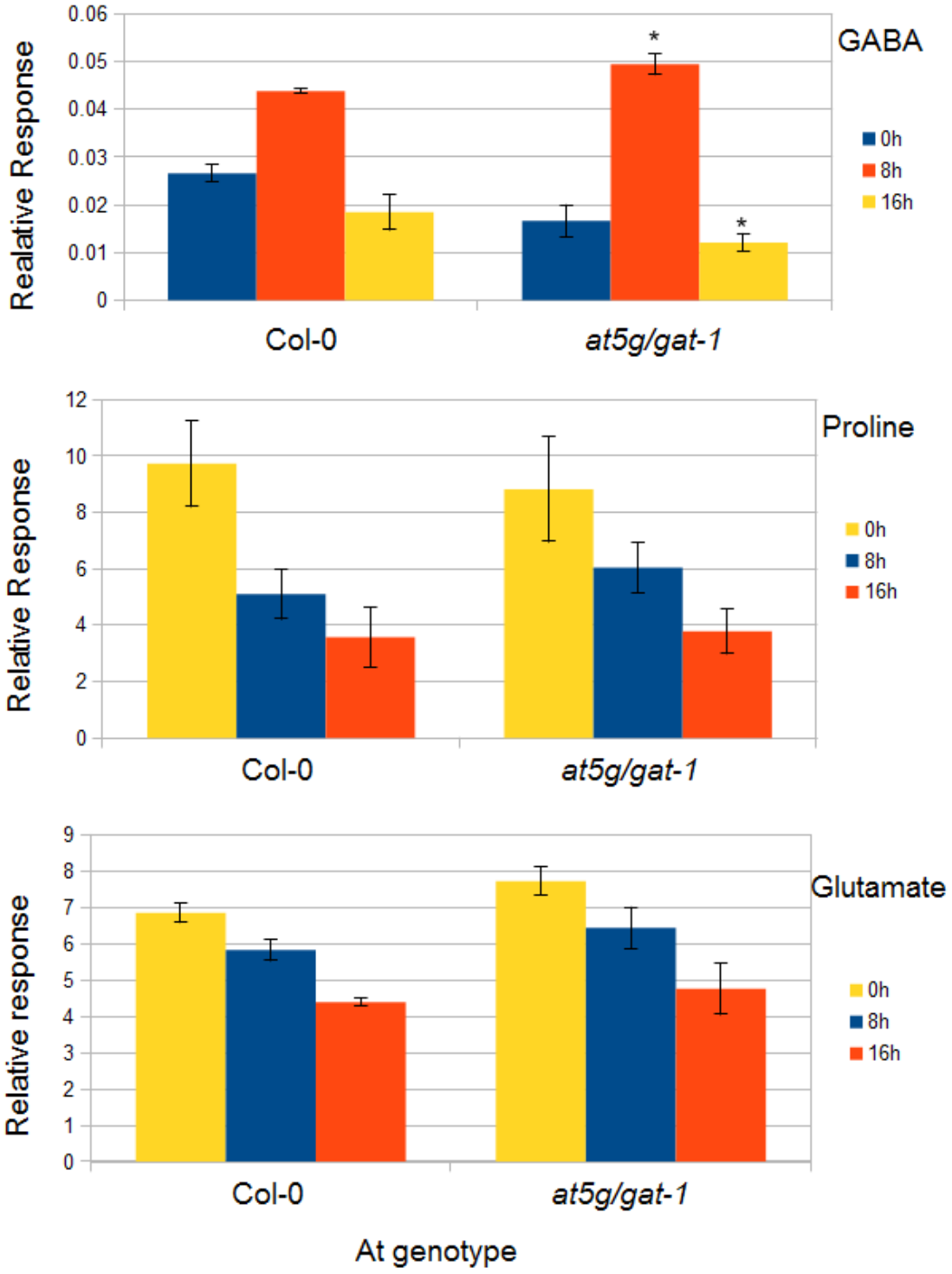


Fig. 30 Amino acid differences between Col-0 and *At-at5g41800/gat-1*. The graphs show the relative responses for GABA, Proline and Glutamate found in Col-0 and *At-at5g41800/gat-1* (labelled at5g/gat-1) treated with PstD *hrpA*- at 8 and 18hpi. The relative responses shown are the average of 3 samples each containing 3 leaves from two plants. The samples taken at 0h were taken from uninfected plants. The error bars indicate standard deviation and asterisks indicate significant differences as compared to Col-0 ($p<0.05$) calculated using Student's t-test.

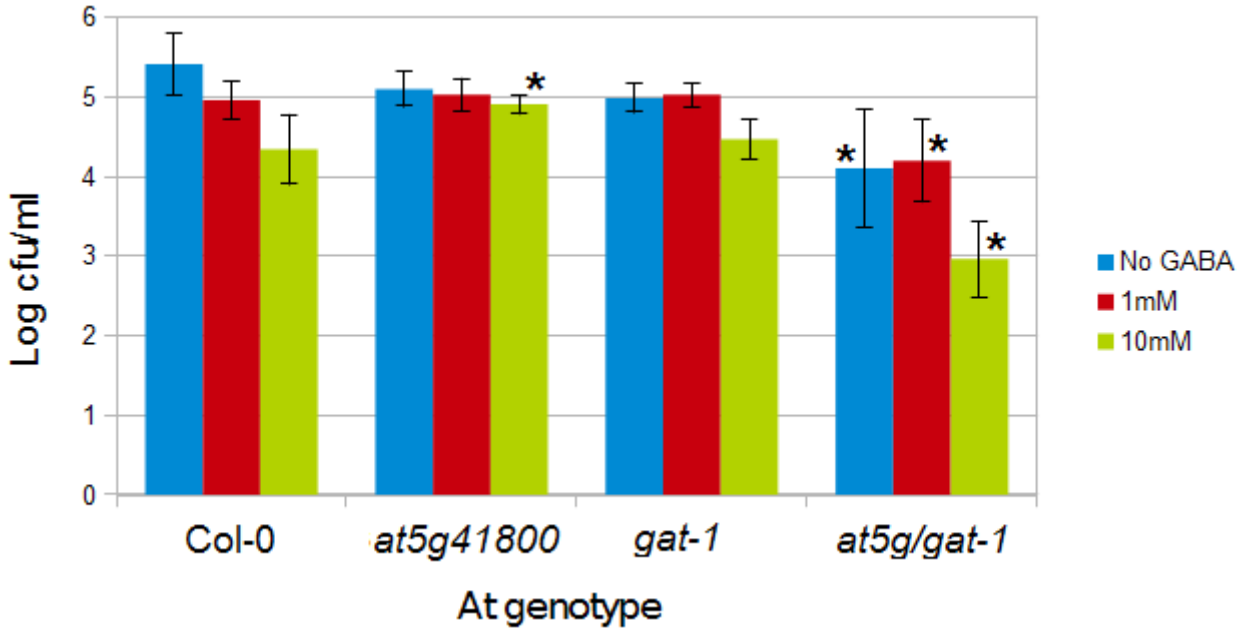


Fig. 31 Log cfu/ml of PstD growth in plants co-inoculated with 1mM GABA, 10mM GABA and with PstD alone (No GABA). The error bars indicate standard deviation and asterisks indicate significant differences ($p<0.05$) compared to Col-0 calculated with Students t-test.

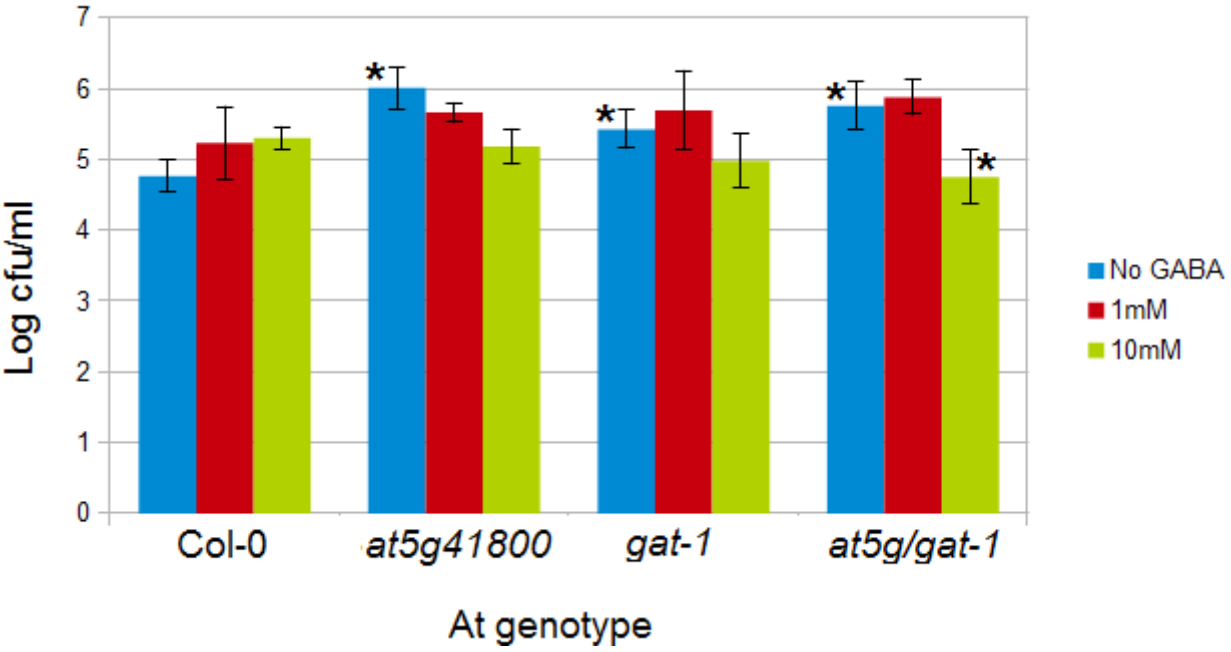


Fig. 32 Log cfu/ml of PstD growth in plants co-inoculated with 1mM GABA, 10mM GABA and with PstD alone (No GABA). The error bars indicate standard deviation and asterisks indicate significant differences ($p<0.05$) compared to Col-0 calculated using Student's t-test.

4: Discussion

4.1: Yeast-two-hybrid Bait Creation

As shown in Fig. 18 creation of pGBKT7-JAZ constructs was successful, although we had originally planned to use all the JAZ10 splice variants upon sequencing we discovered that the JAZ10.4 variant was actually JAZ10.3 so we dropped this splice variant. This is the reason why JAZ10.4 is present in Fig. 13 and Fig. 15 but is not mentioned thereafter. Throughout the bait construction process PCR results were consistent with the expected sizes of the JAZ sequences we PCR'd.; JAZ5 - 825bp JAZ7 - 447bp JAZ10.1 - 594bp JAZ10.3 – 558bp (see Fig. 13, Fig. 15, Fig. 16 and Fig. 18)[165]. After the bait sequences had been ligated into pGBKT7 vectors the cloned vectors were checked using enzyme digestion and sequencing (see Fig. 17 and Appendix 5.2 respectively). The enzyme digestion and sequencing results indicated that the bait sequences had been correctly cloned into the vectors so we transformed them into *S. cerevisiae* Y2HG as per the Yeastmaker 2 manual. Colony PCR confirmed the presence of the inserts (see Fig. 18), a western blot confirmed that the proteins were being expressed with a MYC tag (see Fig. 19). We expected bands for each sample tested at the following kDa's; pGBKT7-53 – 57kDa, pGBKT7-JAZ5 – 52kDa, pGBKT7-JAZ7 – 38.9kDa pGBKT7-JAZ10.1 – 43.8kDa pGBKT7-JAZ10.3 – 42.5kDa. As seen in Fig. 19 the bands for pGBKT7-JAZ5 give a much stronger signal than the other JAZ constructs, furthermore the bands do not appear to correctly correspond with the protein marker bands. These issues were not seen to affect our interpretation of the western blot as subsequent sequencing of the vectors gave the expected sequences. We also confirmed that expression of our bait proteins was not toxic in *S. cerevisae* Y2HG by comparing growth of transformed Y2HG with Y2HG transformed with empty pGBKT7 vectors. Furthermore the Y2HG transformed with our bait vectors did not auto activate reporter genes. The results of the control experiments combined with the results of the sequencing and western blot indicated that our sequences were correctly cloned into pGBKT7 and transformed into *S. cerevisae* Y2HG.

4.2 Yeast-two-hybrid prey library creation

We constructed our prey library using three different sets of RNA species that had been created previously. We checked the integrity of the library using Nanodrop (Agilent)

and RNA gel electrophoresis (see Table 8 and Fig. 20). The absorbance at 260nm/280nm and 260nm/230nm were well within acceptable limits and RNA gel electrophoresis indicated good quality RNA. The gel electrophoresis of the samples generated by the LD-PCR reaction matched the indicative picture in the manual and showed nothing unexpected. Following purification of the LD-PCR reactions using Chroma spin TE-400 columns we analysed the cDNA libraries using Nanodrop (Agilent) and the values obtained indicated we had obtained sufficient quantities and purities of cDNA (see Table 9). The concentrations were within the expected 2-5µg cDNA and the absorbances measured were acceptable. Upon transformation of the cDNA libraries into *S. cerevisae* Y187 we did not obtain 1 million independent clones as mandated by the manual, although our hrpA library transformation came closest at 990000 independent clones (see Table 10.). As we did not have >1 million independent clones in our library, it was not as complex as recommended by the manual so possible interactors may not be represented. However the library was within 10% of the recommended minimum so we felt that the library was complex enough to suit our purposes. A higher complexity library would allow us to detect interactions between JAZs and proteins with low representation in the transcriptome. The reason for the large range in independent library clones between DC3000 and Mixed is because we used two plates to measure the number of clones. Therefore we calculated the complexity of the library using values from both. In future work we could remake the libraries so they are of suitable complexity, however that was not possible for this project as we did not have enough RNA left to produce the cDNA needed for additional libraries nor the money.

4.3 Yeast-two-hybrid screenings

Before we could perform a yeast-two-hybrid screening using our own cDNA library and prey we had to perform control screenings to ensure that we could successfully mate the two yeast strains (Y2H Gold and Y187) as well as ensure that the drop out media was performing correctly. Fig. 22 shows the results of our control mating where it can clearly be seen that the drop out media was correctly selective. Diploid Y2HG-pGBKT7-53 x Y187-pGADT7-T produced blue colonies in the presence of X-α-Gal and grew on media containing AbA (see Fig. 22) whereas the Y2HG-pGBKT7-Lam x Y187-pGADT7-T diploids did not grow on media containing AbA. The library

screening was performed using Y2HG-pGBKT7-JAZ5 against Y187 containing the DC3000 library (see 3.3). The number of screened clones and the mating efficiency (see Table 11) were determined according to the Matchmaker manual (see Appendix 5.1). The manual states if less than 1 million diploids are screened and the mating efficiency is less than 2%, genuine interactions on media containing AbA have a much lower chance of being detected. The number of independent clones required for a good screen will depend on the complexity of the genome used to create the library. Clontech assured us that the reason for stating a minimum of 1 million independent clones was to ensure some rare transcripts were represented in the library being screened. The data in Table 11 shows we were well above the required minimum for both independent clones screen and the over all mating efficiency. However, the Matchmaker Gold Yeast-two-hybrid system manual (Clontech) states that overall mating efficiencies of 2-5% are readily achievable which causes some concern about whether our numbers accurately reflect the mating efficiency as they are so high. Our media correctly selected for diploid Y2HG-pGBKT7-53 x Y187-pGADT7-T, as these diploids formed colonies but Y2HG-pGBKT7-Lam x Y187-pGADT7-T did not. This indicates that the media was correctly selective for diploids containing both pGBKT7 and pGADT-7 implying that our mating efficiencies were correct.

We plated blue diploid colonies onto higher stringency SD/-Ade/-His/-Trp/-Leu/X/AbA media and performed colony PCR on the surviving colonies, the results of which are shown in Fig. 23 and Fig. 24. Colony PCR confirmed that prey inserts in pGADT7 vectors were present in the colonies tested, as there appeared to be some variation in inserts sizes we purified the plasmids from the colonies and sequenced them. As shown in Table 12, six of the seven interactors were aligned to Heat shock cognate protein 70-1 (HSC70-1), with one interactor showing alignment to GSTF10. GSTF10 is Glutathione S-transferase 10 a member of the phi class of GST's which functions in glucosinolate biosynthesis.[166] GSTF10 has been shown to be upregulated by MeJA, Et (but not SA) and by avirulent PstD *AvrRpt2* but down regulated by MYC2.[166–168] Interestingly GSTF10 has been shown to interact with BAK1 which has important functions in the brassinosteroid signalling pathway and activation of ETI, the PstD effector AvrPtoB directly inhibits BAK1 to prevent ETI.[169,170] Brassinosteroids have been shown to regulate JA signalling, for example JA induced anthocyanin production is enhanced by brassinosteroids while JA induced root growth inhibition is negated by

brassinosteroids.[171] GSTF10 has also been shown to interact with SA which inhibits GSTF10 activity.[172] The redox state of the cell is largely controlled by GSH concentration and the GSH:GSSG (oxidized GSH) ratio, therefore increases in GSH and inhibition of GSTF activity would increase the reductive state of the cell.[172] Studies show that pathogen infection (which also results in increased SA levels) increase GSH content and the ratio between GSH and GSSG.[173] *Tian et al,. 2012* suggests that this indicates a possible role for GSTF10 in disease resistance as increases in SA and therefore increased GSTF inhibition would alter the ratio between glutathione (GSH) and its oxidized state (GSSG) causing an increased reductive cell state which is necessary for NPR1 activation (see 1.2.5).[172]

HSC70-1 is a chaperone protein located in the cytosol and nucleus involved in control of protein homeostasis. HSC70-1 mainly binds nascent and stress induced polypeptides, non-native proteins and folding intermediates.[174–176] There are fourteen *HSC70* genes in *At, HSC70-1* through *HSC70-5* express functionally redundant but essential proteins (*hsc70* null mutants are embryo lethal).[174,175] *HSC70-1* overexpression leads to ABA hypersensitivity and compromised stomatal closure in response to ABA and to flg22 perception.[174] *HSC70-1* expression is upregulated in *At* during PstD challenge and although *hsc70* mutants do not exhibit defence phenotypes, *HSC70-1* overexpression compromises *At* resistance to virulent (PstD) and avirulent PstD AvrRpm1, PstD AvrRps4.[175] Additionally HSC70 is the target of AvrHopI1, a PstD avirulence protein, and is necessary for HopI1 mediated virulence.[177] HopI1 forms complexes with cytosolic HSC70, recruits HSC70 to the chloroplasts and affects HSC70 ATP hydrolysis.[177] HopI1 is known to interfere with chloroplast synthesis of SA and SA accumulation, so HSC70 recruitment may be the mechanism for this interference via HSC70 interference with folding or complex assembly of SA biosynthesis or transport components.[177] Although HSC70-1 does not function in JA signalling, HSC70-1 is a co-chaperone of SGT1 and has been shown to associate with SGT1 *in vivo* in the cytosol and the nucleus.[175] *At sgt1b* mutants are compromised in SCF^{COI1} activity and as a result they are unresponsive to coronatine, in this way SGT1 is necessary for JA signalling.[178]

The interactions between HSC70-1 and GSTF10 with JAZ5 can be interpreted in a number of ways, 6/7 clones were identified as HSC70-1 so it is possible that the binding is a mere artefact and that the interactions mean nothing. However we find it curious

that both of the interactors that we identified have roles in plant defence and these roles are related to SA. GSTF10 is hypothesized to change cellular redox state to allow for NPR1 activation while HSC70 is targeted by HopI1 to interfere with SA biosynthesis or transport in the chloroplasts.[172] We have not been able to find any other authors reporting these same interactions, suggesting potential novelty, so further experimentation is needed validate these results. As no other researchers have specifically screened PstD induced infection libraries, it remains a possibility that these are indeed genuine interactors and that these interactions only occur in the context of the infection transcriptome. These interactions also highlight the need to exhaustively map JAZ interaction partners to gain further insight into the individual and collective function of JAZ proteins. As work for the future we could check whether the interactions were false positives, by ensuring that these specific preys could not activate the reporter genes by itself and furthermore we would confirm the interactions occur *in vivo* using coimmunoprecipitation. Additionally if we could screen against libraries of suitable complexity it would be possible to compare and contrast protein-protein interactions between PtsD and PtsD *hrpA* infected plants, which might illuminate proteins that have roles in defence against virulent and avirulent pathogens. Screenings using our other bait proteins would also be valuable as protein interactions between the JAZs could be contrasted which would help further our understanding of the individual roles of the JAZs.

4.4: JAZ mutant susceptibility to B cinerea.

Previous work by Garoufalia Eleftheriadou had shown that *At-jaz5/10* mutants were more resistant to *B. cinerea* than the wild type Col-0. We sought to expand upon that work by testing some different *At-jaz* mutants. As seen in Fig. 25 and Fig. 26 the single *At-jaz* knockouts do not display any significant differences in resistance to *B. cinerea* as compared to wild type. Significant differences ($p < 0.05$) are only seen when two *At-jaz* genes are knocked out, as in *At-jaz5/7* and *At-jaz7/10* which had increased average lesion area compared to Col-0. These results are very interesting as JAZ7 and some JAZ10 splice variants lack Jas domains that are necessary for JAZ binding to COI1. It has been hypothesized that JAZs lacking COI1 interaction are responsible for dampening JA responses to prevent runaway JA responses (see 1.2.1.6). *B. cinerea* is a necrotrophic pathogen so the induction of JA/Et pathways is vital to *At* (see 1.2.2 and

1.4.2), as SA signalling leading to HR will only benefit the pathogen. JA/Et signalling is antagonistic to SA signalling so the removal of JAZ7 and JAZ10 splice variants, that are JA signalling inhibitors, could strengthen the JA response. We believe that the removal of JAZ7 and JAZ10 is especially important because they are resistant to JA mediated degradation via SCF^{COI1} (see 1.2.16). It does however remain very curious why *At-jaz5/7/10* did not exhibit lowered resistance to *B. cinerea* as both *At-jaz5/7* and *At-jaz7/10* mutants did show significantly decreased resistance compared to Col-0. It would seem that increased deregulation of JA responses would yield heightened resistance to *B. cinerea* but the *At-jaz5/7*, *At-jaz7/10* and *At-jaz5/7/10* mutants do not show this. It has been previously shown that JAZ10 has an effect on resistance to *B. cinerea* so perhaps the knocking out of JAZ10 in the triple mutant abolishes the negative effect of *At-jaz5/7* on plant resistance.[179] This may be due to the alternatively spliced JAZ10.4 protein, which is highly resistant to SCF^{COI1}-26S mediated degradation, curtailing JA responses (see 1.2.16) needed to fight *B. cinerea* infection.[29] Further investigation into the *At-jaz5/7/10* mutant and other JAZ triple mutants could yield significant insight into the JA signalling pathway.

The data for Fig. 25 and Fig. 26 was compiled from the leaves shown in Fig. 27. From the image we can see that the JAZ mutants suffer from extensive chlorosis when compared to Col-0. Especially *At-jaz5/7* and *At-jaz7/10* where entire leaves are chlorotic. The image also shows how the triple mutant *At-jaz5/7/10* appears far healthier than the double mutants or even single mutants, apart from *At-jaz7* that has the lowest lesion sizes of the mutants tested, other than Col-0. Although no conclusions can be drawn until the experiments are repeated, we did not repeat the experiments as we ran out of time in the laboratory. We do not believe that the method is the source of the inconsistent results as similar methods are commonly used.[63,151,155] However in the future we would ensure that further repeated experiments were performed with great care to get more consistent results.

We believe that further investigation is warranted by these results and in future work expanding the range of JAZ mutants could yield interesting results. If other double and triple JAZ mutants could be generated it may be possible to identify which JAZs are most critical for resistance to *B. cinerea.* For instance *At-jaz7/8* could be highly susceptible to *B. cinerea* if runaway JA signalling is responsible for the lowered resistance we see in *At-jaz5/7* and *At-jaz7/10,* however given the results of the *At-*

jaz5/7/10, At-jaz7/8 may show increased resistance due to a higher JA response.

4.5: Amino Acid Transport mutants

Previous work had shown differences in GABA levels following infection with PtsD. To expand on this work we performed two experiments in which we infiltrated *At* amino acid transporter mutants with PstD, sampled them at 8hpi and 16hpi.

In comparing the results from Fig. 28 and Fig. 29 it should be noted that there is a two hour difference in end time point. This was done in order to see whether amino acid levels would be affected by the extra time the plants were infected. In Fig. 28 all of the measured relative responses for GABA are significantly lower than those measured in Col-0, except 8hpi and 16hpi for *At-at5g41800/gat-1*. We see this trend continued in Fig. 29 but with a number of caveats; 8hpi *At-gat-1*, 0h *At-at5g41800*, all *At-at5g41800/gat-1* and *At-prot-1* time points. It is difficult to speculate on the nature of these differences as we do not have more data, but the response for GABA in *At-at5g41800* is so large that it may be the result of leaf damage or another source. It has previously been shown that GABA levels are elevated in response to wounding.[180–182] Additionally the fall in GABA that we see in Fig. 28 over the time course is reversed in Fig. 29. However there are some consistencies, for example *At-at5g41800/gat-1* gives a significantly higher relative response for GABA compared to *At-at5g41800* in both experiments. For proline we see a consistent fall in relative response for Col-0 across the time course and although the pattern for *At-gat-1* remains the same (decrease from 0h to 8hpi then increase from 8hpi to 18hpi) only one time point shows significantly less proline than Col-0. *At-at5g41800* displays significantly decreased proline compared to Col-0 across all time points in both Fig. 28 and Fig. 29 and compared to *At-gat-1* at 18hpi. The double mutant *At-at5g41800/gat-1* displays a similar pattern in both Fig. 28 and Fig. 29, at 18hpi *At-at5g41800/gat-1's* relative response is significantly larger than Col-0's. *At-at5g41800/gat-1* gave significantly higher relative responses than *At-at5g41800* at all time points as well as *At-gat-1* at 0h and 8 hpi in both experiments. Fig. 28 and Fig. 29 both show decreases in glutamate as the time course progresses but the significant differences in relative response for *At-gat-1* are not present in Fig. 29. However the significantly larger response for glutamate in *At-at5g41800/gat-1* compared to Col-0 remains at 0h in both figure. The significantly larger relative response of *At-at5g41800/gat-1* compared to *At-at5g41800/gat-1* at 18hpi is consistent

in both experiments.

It would be best if we had more experiments using both time points to more gain further confidence in our data, as such we did perform other experiments but due to problems with our freeze dryer these samples were lost so the experiments could not be completed. We believe that there is some value in our data as there are consistencies between the two experiments. Given more time we would perform further repetitions of these experiments to confirm the data. Further work could be conducted on comparisons between apoplastic fluid and cellular amino acid content, as the amino acid transporters here transport amino acids from the apoplast and into the cell. If there were significant differences in the amino acid content the apoplastic fluid of the transport mutants compared to Col-0, then differences in growth of PstD may be a result of the mutants not meeting the nutritional requirements of the pathogen in the apoplast.

To draw any real conclusions from this data we need more repetitions to establish an real pattern for perturbations in amino acid levels during the time course. The data from Fig. 28 and Fig. 29 conflicts so it is difficult to come to any real conclusions.

We had previously shown that *At-at5g41800/gat-1* was significantly more resistant to PtsD than Col-0 although it showed greatly increased chlorosis. Recently it was shown that GABA lowers the susceptibility of *At* to PtsD so we performed experiments where we co-inoculated PtsD and GABA into the abaxial surface of *At* leaves. Fig. 31 and Fig. 32 show the results of these experiments. In Fig. 31 we can see that *At-at5g41800/gat-1* supported significantly less growth of PtsD compared to Col-0 for all the treatments. We had previously shown that growth of PtsD in *at5g41800/gat-1* was significantly reduced compared to Col-0. We also observed significantly increased growth in *At-at5g41800* as compared to Col-0. Additionally it should be noted that when comparing the growth of PtsD alone to the growth of PtsD co-inoculated with 10mM GABA in the same genotype, there is significantly less growth ($p<0.05$) for Col-0, *At-gat-1* and *At-at5g41800/gat-1*. Fig. 32 displays the results of a repeat of the experiment in Fig. 31, but here we can see that the trend for Col-0 is reversed. In Fig. 32 we can see that the log cfu/ml of the *At* mutants is significantly increased compared to Col-0 for plants inoculated with PstD only. We have not observed these significant differences before which does imply that an error occurred when the experiment was performed. However *At-at5g41800/gat-1* still shows significantly decreased growth for PtsD co-inoculated with 10mM GABA as compared to Col-0. Also *At-at5g41800* and *At-at5g41800/gat-1*

treated with PtsD and 10mM GABA have significantly reduced growth compared to treatments with PtsD alone. Between Fig. 31 and Fig. 32 we cannot explain why the trend for Col-0 has been reversed while the trend for the mutants has largely stayed the same, more repeats of the experiment are needed to clarify which trend is correct.

At-at5g41800/gat-1 treated with PtsD and 10mM GABA retained significantly lowered growth compared to Col-0 in both experiments. Furthermore *At-at5g41800/gat-1* treated with PtsD and 10mM GABA displayed significantly lowered growth compared to *At-At-at5g41800/gat-1* treated with PtsD alone in both experiments. Therefore we believe that this work justifies further investigation into the effect of GABA on PtsD growth in these mutants as well as Col-0.

4.6: Self Reflection and Future Work

To conclude we feel that the ideas and rationales behind the project were well thought out and gave opportunity for great results. Unfortunately the execution of the project was not entirely successful, identification of two putative interactors is exciting but there was certainly room for the discovery of many more interactors than that. We had not worked with the Clontech system before nor did we any experience working with *S. cerevisae* which caused some issues. If we were to run a Yeast two hybrid project again it would be a lot easier due to the experience gained from this project. The main issues with the work carried out are lack of repeat experiments which was mainly due to lack of time. This is obviously quite a grievous issue as none of the data can be heavily relied upon because of the lack of repeats to increase confidence in the data. We feel that although the work has issues at the very least it provides a solid foundation for future work. The bait constructs can be used for future work in identifying protein-protein interactors with JAZ5, JAZ7, JAZ10.1 and JAZ10.3. We feel that there is a great deal of worth in exploring the different protein-protein interactions that arise under different pathogen stresses, especially when interactions can be compared to interactions that occur during infection with avirulent PtsD. The work with *B. cinerea* yielded interesting results that can be further investigated to elucidate the roles of individual JAZs and their roles in combination with one another. The GABA co-inoculation work can certainly be explored further, it has already been shown that GABA acts both as a source of nutrition for PtsD and a growth inhibitor. The project was also invaluable in terms of research experience as well as growth and development as a researcher.

There are still a great many issues to be resolved for JA signalling, the individual functions of the JAZs have yet to be elucidated also how hetero/homodimerization plays a part in controlling JAZ degradation by the SCF^{COI1}-26S proteasome pathway. There is also a need to examine how varying JA levels induce degradation of different JAZs, how JA levels affect assembly of the JAZ-COI1 receptor and determination of JA levels throughout the plant in response to abiotic/biotic stimulus. Modelling JA levels spatially and temporally in different cell types would also help our understanding of how JA is distributed and transported throughout the plant. Even JA biosynthesis has yet to be completely understood as the signal responsible for the activation of plastid lipases that free α-linoleic is unknown. The work to further our understanding of JA signalling is very important as it is a major player in plant defence, so advances in our knowledge could be put to great use in agriculture and securing the worlds food supply.

5: Appendix

5.1: Clontech Protocols

The Clontech manuals used in the project are as follows;

Matchmaker Gold Yeast-two-hybrid System User Manual
(http://www.clontech.com/FR/Products/Protein_Interactions_and_Profiling/Yeast_Two-Hybrid/ibcGetAttachment.jsp?cItemId=17597&fileId=5877852&sitex=10023:22372:US)

Make Your Own “Mate & Plate” Library System User Manual
(http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17598)

In-Fusion HD Cloning Kit User Manual
(http://www.clontech.com/FR/Products/Cloning_and_Competent_Cells/Cloning_Kits/ibcGetAttachment.jsp?cItemId=17497&fileId=6518811&sitex=10023:22372:US)

Yeastmaker Yeast Transformation System 2 User Manual
(http://www.clontech.com/FR/Products/Protein_Interactions_and_Profiling/Yeast_Two-Hybrid/ibcGetAttachment.jsp?cItemId=17583&fileId=5877836&sitex=10023:22372:US)

Yeast Protocols Handbook
(http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17602&minisite=10023&secItmId=16124)

Matchmaker Monoclonal Antibodies User Manual
(http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17581&minisite=10023&secItmId=14865)

We have created a zip file of all the manuals used that can be downloaded here:
https://docs.google.com/file/d/0B1NgzredCy7YSTJzRTJ1bmQtcjg/edit?usp=sharing

5.2: DNA Sequences

The sequences that we received from sequencing our bait proteins as described in section 3.1 are as follows:

JAZ5:

GCTTAGGGAGACAGAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGAGGCCGAATTCATGTCGTCGAGCAAT
GAAAATGCTAAGGCACAAGCGCCGGAGAAATCTGACTTTACCCGGAGATGTAGTTTGCTCAGCCGTTACTTGAAGGA
GAAGGGTAGTTTCGGAAACATTGATCTTGGCTTATACCGAAAACCCGATTCCAGTCTCGCGTTGCCCGGAAAATTCG
ATCCACCAGGGAAACAAAATGCGATGCATAAGGCAGGGCATTCCAAAGGCGAACCCTCTACCTCATCAGGAGGCAA
AGTCAAAGATGTTGCTGACCTCAGTGAATCACAGCCAGGAAGTTCGCAGCTGACCATATTCTTCGGAGGGAAAGTTT
TAGTATATAATGAGTTCCCCGTAGACAAAGCTAAAGAGATTATGGAAGTAGCAAAACAAGCCAAGCCTGTGACTGAGA
TTAACATTCAGACACCAATCAATGACGAAAACAACAACAACAAGAGCAGCATGGTTCTTCCTGATCTCAATGAGCCTA
CTGATAATAATCACCTAACAAAGGAACAACAACAGCAACAAGAACAAAATCAGATCGTGGAACGTATAGCACGTAGAG
CTTCCCTCCATCGATTCTTTGCTAAACGGAAAGACAGAGCTGTGGCTAGGGCTCCGTACCAAGTTAACCAAAACGCA
GGTCATCATCGTTATCCTCCCAAGCCAGAGATTGTAACCGGTCAACCACTAGAGGCAGGACAGTCGTCACAAAGAC
CGCCGGATAACGCCATTGGTCAAACCATGGCCCATATCAAATCAGACGGTGATAAAGATGATATTATGAAGATTGAAG
AAGGCCAAAGTTCGAAAGATCTCGATCTAAAGCTATAGGGATCCGTCGACCTGCAGCGGCCGCATAACTAGCATAAC
CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCGCGCTTGCAGCCAAGCTAATTCCGGGCGAATTTCTTAT
GATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAAAATAAGTGTATACAAATTTTAAAGTGACTCTTACGTTTTAAAACG
AAAATTCTTATCTGAGTACTCTTTCCTGTAGTCAGTTGCTTTCTCAAGTATAGCATGAGGTCGCTCTATTGACCACACC
TCTACGGCATGCAAGCTTGGCGTAATCATGGTCAATAGCTGTTTCCTGTGTGGAAATGTATCCGCTCACAATCCACCA
CACATACGAGCCGAAGCATAAAGGTGTTAAGCCTGGGGTGCCTATGAGTGAGCTAACTCAATTATGCCTGGCGCCTC
ACTGCCCGCTCCAGTCGGAACTGTCAGGCAACTGCATAATGATCGTCAATCGCGGCGAAGCGATGCTATGGCCCTT
ACGCTTCTTCACTCAAGTGACCCTCGT

JAZ7:

GCTATGGAGGACAGAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGAGGCCGAATTCATGATCATCATCATCA
AAAACTGCGACAAGCCTTTACTCAATTTCAAAGAGATGGAGATGCAAACAAAATGCGACTTGGAACTTCGCCTTCTTA
CTTCTTCTTATGATTCTGATTTCCATAGCTCGTTGGACGAATCAAGCAGCTCTGAAATTTCACAACCAAAGCAAGAATC
TCAGATATTAACCATTTTCTACAACGGGCACATGTGTGTTTCTTCAGATCTTACCCATCTTGAGGCTAACGCTATACTAT
CGCTAGCGAGTAGAGATGTGGAAGAGAAATCTTTATCCTTGAGAAGTTCAGACGGTTCGGATCCTCCAACAATCCCA
AACAATTCGACTCGATTTCATTATCAAAAGGCCTCTATGAAGAGATCTCTTCACAGTTTTCTTCAGAAACGAAGTCTTC
GGATTCAAGCAACTTCCCCTTACCACCGTTACCGATAGGGATCCGTCGACCTGCAGCGGCCGCATAACTAGCATAAC
CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCGCGCTTGCAGCCAAGCTAATTCCGGGCGAATTTCTTAT
GATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAAATAAGTGTATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACGA
AAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTATAGCATGAGGTCGCTCTTATTGACC
ACACCTCTACCGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAAT
TCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGG
GGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGC
GGCGAGCGTATCAGCTCACTCAAAGCGGTAATACGGTTATCCACAGAATCAGGGATAACGCAGAAAGAACATGTGAG
CAAAAGGCCAGCAAAAGCCAGACCGTAAAAGCCGCGTTGCTGGCGTTTTCATAGCTCGCCCCCCTGACGAGCATCC
AAAAATCGAGGCTCAGTCAGAGGTGGCGAAACCCGACAGGACTATAAGAATCAGGCGTTCCCCTGAAGCCTCCTTC
GTGCGCCTCCTCTGTCGACCTGCCCTAACCGGGATTCCTGTTCCGCCTTCCTTCGAAGCGTGGGCCTTTCTCTAGC
TCAGCGTTAGGATTCCAGTTCCGGTTTATAGTCGTAGCGTCTCCAAAGCCTAGAGGC

JAZ10.1:

GCTTTTGCAGAGCAGAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGAGGCCGAATTCATGTCGAAAGCTAC
CATAGAACTCGATTTCCTCGGACTTGAGAAGAAACAAACCAACAACGCTCCTAAGCCTAAGTTCCAGAAATTTCTCGA
TCGCCGTCGTAGTTTCCGAGATATTCAAGGTGCGATTTCGAAAATCGATCCGGAGATTATCAAATCGCTGTTAGCTTC
CACTGGAAACAATTCCGATTCATCGGCTAAATCTCGTTCGGTTCCGTCTACTCCGAGGGAAGATCAGCCTCAGATCC
CGATTTCTCCGGTCCACGCGTCTCTCGCCAGGTCTAGTACCGAACTCGTTTCGGGAACTGTTCCTATGACGATTTTC
TACAATGGAAGTGTTTCAGTTTTCCAAGTGTCTCGTAACAAAGCTGGTGAAATTATGAAGGTCGCTAATGAAGCAGCA
TCTAAGAAAGACGAGTCGTCGATGGAGACAGATCTTTCGGTAATTCTTCCGACCACTCTAAGACCAAAGCTCTTTGG
CCAGAATCTAGAAGGAGATCTTCCCATCGCAAGGAGAAAGTCACTGCAACGTTTTCTCGAGAAGCGCAAGGAGAGA
TTAGTATCAACATCTCCTTACTATCCGACATCGGCCTAAGGATCCGTCGACCTGCAGCGGCCGCATAACTAGCATAAC
CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCGCGCTTGCAGCCAAGCTAATTCCGGGCGAATTTCTTAT
GATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAAATAAGTGTATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACGA
AAATTCTTATTCTTGAGTACTCTTTCCTGTAGTCAGTTGCTTTCTCAGTATAGCATGAGGTCGCTCTTATTGACCACAC
CTCTACCGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCA
CACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAACTAACTCACATTAATTGCGTT
GCGCTCCCTGCCCGCTTTCCAGTCAGGAAACCTGTCGTGCCAGCTGCATATGAATCGGCCCACGCGCGGGGAGAA
GCGGTTTGCGTATTGGGCGCCTCCGCTTCCTCGTCAATGACTCACTGCACCTGGTCGTCGCTGCGGCGAGCGGTAT
AAGCTTCATTCAAAGCGGTAATACCGTTTTCCCTCAGAATCCGTGAAAACCCTAGTAAGAACTAGGTGCTAGACGCAT
GCATAAAGCTCATGAGCCGTATAAAGGACGAGCATGCCTTGTCGATTAATACGATAAGCTCCAGTCACCTTGATCGCA
ATCATGCCCAGAACATTGTCTCGACTAAGTCATCAGAGAGTGCGTCGAAACTCCTATAGCAGTCCAGTTACATAGGAC
TCTCTCGGAATACTGAT

JAZ10.3:

GAATTGGGCAGACGAGCTGATCTCAGAGGAGGACCTGCATATGGCCATGGAGGCCGAATTCATGTCGAAAGCTACC
ATAGAACTCGATTTCCTCGGACTTGAGAAGAAACAAACCAACAACGCTCCTAAGCCTAAGTTCCAGAAATTTCTCGAT
CGCCGTCGTAGTTTCCGAGATATTCAAGGTGCGATTTCGAAAATCGATCCGGAGATTATCAAATCGCTGTTAGCTTCC
ACTGGAAACAATTCCGATTCATCGGCTAAATCTCGTTCGGTTCCGTCTACTCCGAGGGAAGATCAGCCTCAGATCCC
GATTTCTCCGGTCCACGCGTCTCTCGCCAGGTCTAGTACCGAACTCGTTTCGGGAACTGTTCCTATGACGATTTTCT
ACAATGGAAGTGTTTCAGTTTTCCAAGTGTCTCGTAACAAAGCTGGTGAAATTATGAAGGTCGCTAATGAAGCAGCAT
CTAAGAAAGACGAGTCGTCGATGGAGACAGATCTTTCGGTAATTCTTCCGACCACTCTAAGACCAAAGCTCTTTGGC
CAGAATCTAGAAGGAGATCTTCCCATCGCAAGGAGAAAGTCACTGCAACGTTTTCTCGAGAAGCGCAAGGAGAGGT
AAGGATCCGTCGACCTGCAGCGGCCGCATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTT
TTGCGCGCTTGCAGCCAAGCTAATTCCGGGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAA
TAAGTGTATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTGGTCA
GGTTGCTTTCTCAGTATAGCATGAGGTCGCTCTTATTGACCACACCTCTACCGGCATGCAAGCTTGGCGTAATCATGG
TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAA
GCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACC
TGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTT
CCTCGCTCACTGACTCGCTGCGCTCGGTCGTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAGCGTAATACGGTTA
TCCACAGAATCAGGGAATACGCCAGAAAGAACATGGTGAGCTAAGCCAGCAAAGCCCAGACCGTTAAAGCCCGATG
CTGGCATTTCCCATAGCCTCCGCCCCTGACGAGTCATCCAATTCGAAGCTAGTTCAGAGGTGGCGAACGGACGCCA
TTTATAGGAATATACAGCCCTTCCCCATGGAAAGCTTCCTG

These are the sequences we received from sequencing our seven putative interactors as described in 3.3.

Colony 1:

GCCGATAATACATACGACGCTACCAGATTACGCTCATATGGCCATGGAAGGCCAGTGAATTCCACCCAAGCAGTGGTA
TCAACGCAGAGTGGCCATTATGGCCGGGGCACAAGAAGAAGGTTGAAGCCAAGAACGCTCTCGAGAACTACGCTTA
CAACATGAGGAACACCATCCAAGACGAGAAGATTGGTGAGAAGCTCCCGGCTGCAGACAAGAAGAAGATCGAGGAT
TCTATTGAGCAGGCGATTCAATGGCTCGAGGGTAACCAGTTGGCTGAGGCTGATGAGTTCGAAGACAAGATGAAGG
AATTGGAGAGCATCTGCAACCCAATCATTGCCAAGATGTACCAAGGAGCTGGTGGTGAAGCCGGTGGTCCAGGTGC
CTCTGGTATGGACGATGATGCTCCCCTGCTTCAGGCGGTGCTGGACCTAAGATCGAGGAGGTCGACTAATTTGTTG
GACATTGACCTCTCTCTTTCTCCTATCTCTATCTCTTTTACTTGCTTTTTTTGATCTGTTAAGACTTTTTATGTTGGGCTT
TTTTAAAGAAGCCCATTTTGTGGTGTTTTTTGGTTAGTACTATTTTGAACAATGGTTGGTTCTATACCAGTTTAGCTACG
ATGACGGATAAAATTAAAAGTTTGCCATTTTCTCTCATATATAAAAAAAAAAAAAAAAAAAAAAAAACTTTGCCCCCCCC
CCCCCCCTTAAGGGGGGGGGTTCTATAGGGGACCCCCCGGGCCGGGGGGGGAAATTTTCCTTTTTTTTTTGGG

Colony 2:

GACTAGGTACATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAATTCCACCCAAGCAGTGGTATC
AACGCAGAGTGGCCATTATGGCCGGGGCACAAGAAGAAGGTTGAAGCCAAGAACGCTCTCGAGAACTACGCTTACA
ACATGAGGAACACCATCCAAGACGAGAAGATTGGTGAGAAGCTCCCGGCTGCAGACAAGAAGAAGATCGAGGATTC
TATTGAGCAGGCGATTCAATGGCTCGAGGGTAACCAGTTGGCTGAGGCTGATGAGTTCGAAGACAAGATGAAGGAA
TTGGAGAGCATCTGCAACCCAATCATTGCCAAGATGTACCAAGGAGCTGGTGGTGAAGCCGGTGGTCCAGGTGCCT
CTGGTATGGACGATGATGCTCCCCTGCTTCAGGCGGTGCTGGACCTAAGATCGAGGAGGTCGACTAATTTGTTGGA
CATTGACCTCTCTCTTTCTCCTATCTCTATCTCTTTTACTTGCTTTTTTTGATCTGTTAAGACTTTTTATGTTGGGCTTTT
TTAAAGAAGCCCATTTTGTGGTGTTTTTTGGTTAGTACTATTTTGAACAATGGTTGGTTCTATACCAGTTTAGCTACGAT
GACGGATAAAATTAAAAGTTTGCCATTTTCTCTCATATATAAAAAAAAAAAAAAAAAAAAAAAAACTTGGGCCCCCCCC
CCCCCCTAAAGGGGGGGGGCTTAAAAGGGGACCCCCCCGGCCCCCGGGGAAAAAAGCCCGATTTTTTTTT

Colony 3:

CCATGATATACCATACACACGCTACCAGATTACGCTCATATGGCCATGGAAGGCCAGTGAATTCCACCCAAGCAGTGG
TATCAACGCAGAGTGGCCATTATGGCCGGGGCACAAGAAGAAGGTTGAAGCCAAGAACGCTCTCGAGAACTACGCT
TACAACATGAGGAACACCATCCAAGACGAGAAGATTGGTGAGAAGCTCCCGGCTGCAGACAAGAAGAAGATCGAGG
ATTCTATTGAGCAGGCGATTCAATGGCTCGAGGGTAACCAGTTGGCTGAGGCTGATGAGTTCGAAGACAAGATGAA
GGAATTGGAGAGCATCTGCAACCCAATCATTGCCAAGATGTACCAAGGAGCTGGTGGTGAAGCCGGTGGTCCAGGT
GCCTCTGGTATGGACGATGATGCTCCCCTGCTTCAGGCGGTGCTGGACCTAAGATCGAGGAGGTCGACTAATTTGT
TGGACATTGACCTCTCTCTTTCTCCTATCTCTATCTCTTTTACTTGCTTTTTTTGATCTGTTAAGACTTTTTATGTTGGG
CTTTTTTAAAGAAGCCCATTTTGTGGTGTTTTTTGGTTAGTACTATTTTGAACAATGGTTGGTTCTATACCAGTTTAGCT
ACGATGACGGATAAAATTAAAAGTTTGCCATTTTCTCTCATATATAAAAAAAAAAAAAAAAAAAAAAAAACTTGGGCCCC
CCCCCCCCCCCTAAAGGGGGGGGTTTTAAAGGGGACCCCCCCCCCCCCGGCTGGAAAAAATTCAATTTTTTTTAA

Colony 4:

GCCTTGATATGAGAACAACGCTACCAGATTACGCTCATATGGCCATGTAAGGCCAGTGAATTCCACCCAAGCAGTGG
TATCAACGCAGAGTGGCCATTATGGCCGGGATCACAAGGACCTGATCTTTTGGGGAAGACTATTGAAGAGAGAGGA
CAAGTAGAGCAATGGTTAGACGTTGAGGCTACAAGTTACCATCCACCACTATTGGCTTTAACGCTCAACATTGTCTTT
GCACCACTTATGGGTTTCCCTGCTGATGAGAAAGTTATTAAGGAGAGTGAAGAGAAGCTTGCAGAAGTGCTTGATGT
CTATGAAGCTCAGCTTTCTAAGAACGAATACTTGGCTGGTGATTTTGTGAGTCTAGCTGATTTGGCTCACCTTCCTTT
CACCGAGTATCTTGTTGGTCCTATTGGGAAGGCTCATTTGATCAAAGATAGGAAGCATGTTAGCGCTTGGTGGGATAA
GATTAGTAGCCGTGCTGCGTGGAAGGAGGTTTCCGCTAAGTACTCACTACCTGTTTAAACAAGGAGATGTTGACTTG
GGGGGTTTGTGTGAGCTTTGTGTTCTTTGAGGGAGTTCGTTTTCGGGTCGATTGTCTTGTTGTTTCAATAAGAAACG
GAACTCTGTCTCATTTGTTGTCTCTGGTTTCTTGTATTCTGTAACTTTTCAAGCTTTTCAATAAGAGTTTAAAATCAGAG
TAAAAAAAAAAAAAAAAAAAAAAAAACCTTGCCCCCCCCCCCCCCCCCAAAGGGGGGGGCTTTAAAGGGACCCCCCG
GGGCCGGGCGGGGGTAAATCCAATATTTTT

Colony 5:

GATCGTATAATCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAATTCCACCCAAGCAGTGGTA
TCAACGCAGAGTGGCCATTATGGCCGGGGCACAAGAAGAAGGTTGAAGCCAAGAACGCTCTCGAGAACTACGCTTA
CAACATGAGGAACACCATCCAAGACGAGAAGATTGGTGAGAAGCTCCCGGCTGCAGACAAGAAGAAGATCGAGGAT
TCTATTGAGCAGGCGATTCAATGGCTCGAGGGTAACCAGTTGGCTGAGGCTGATGAGTTCGAAGACAAGATGAAGG
AATTGGAGAGCATCTGCAACCCAATCATTGCCAAGATGTACCAAGGAGCTGGTGGTGAAGCCGGTGGTCCAGGTGC
CTCTGGTATGGACGATGATGCTCCCCTGCTTCAGGCGGTGCTGGACCTAAGATCGAGGAGGTCGACTAATTTGTTG
GACATTGACCTCTCTCTTTCTCCTATCTCTATCTCTTTTACTTGCTTTTTTTGATCTGTTAAGACTTTTTATGTTGGGCTT
TTTTAAAGAAGCCCATTTTGTGGTGTTTTTTGGTTAGTACTATTTTGAACAATGGTTGGTTCTATACCAGTTTAGCTACG
ATGACGGATAAAATTAAAAGTTTGCCATTTTCTCTCATATATAAAAAAAAAAAAAAAAAAAAAAAAACTTTTCCCCCCCC
CCCCCCTTTAAGGGGGGGGGTCTAAAAGGGGACCCCCCCGCCCCCGGCTGAAAAATATTTTTTAATTTTT

Colony 6:

GCGGAGGTCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAATTCCACCCAAGCAGTGGTATCA
ACGCAGAGTGGCCATTATGGCCGGGGCACAAGAAGAAGGTTGAAGCCAAGAACGCTCTCGAGAACTACGCTTACAA
CATGAGGAACACCATCCAAGACGAGAAGATTGGTGAGAAGCTCCCGGCTGCAGACAAGAAGAAGATCGAGGATTCT
ATTGAGCAGGCGATTCAATGGCTCGAGGGTAACCAGTTGGCTGAGGCTGATGAGTTCGAAGACAAGATGAAGGAAT
TGGAGAGCATCTGCAACCCAATCATTGCCAAGATGTACCAAGGAGCTGGTGGTGAAGCCGGTGGTCCAGGTGCCT
CTGGTATGGACGATGATGCTCCCCTGCTTCAGGCGGTGCTGGACCTAAGATCGAGGAGGTCGACTAATTTGTTGGA
CATTGACCTCTCTCTTTCTCCTATCTCTATCTCTTTTACTTGCTTTTTTTGATCTGTTAAGACTTTTTATGTTGGGCTTTT
TTAAAGAAGCCCATTTTGTGGTGTTTTTTGGTTAGTACTATTTTGAACAATGGTTGGTTCTATACCAGTTTAGCTACGAT
GACGGATAAAATTAAAAGTTTGCCATTTTCTCTCATATATAAAAAAAAAAAAAAAAAAAAAAAAACTTGGCCCCCCCCC
CCCCCCCTAAGGGGGGGGGATTAAAACGGGACCCCCCGGCCCCGGGGGAAAAGACTCTATTTTATTTTTCCG

Colony 7:

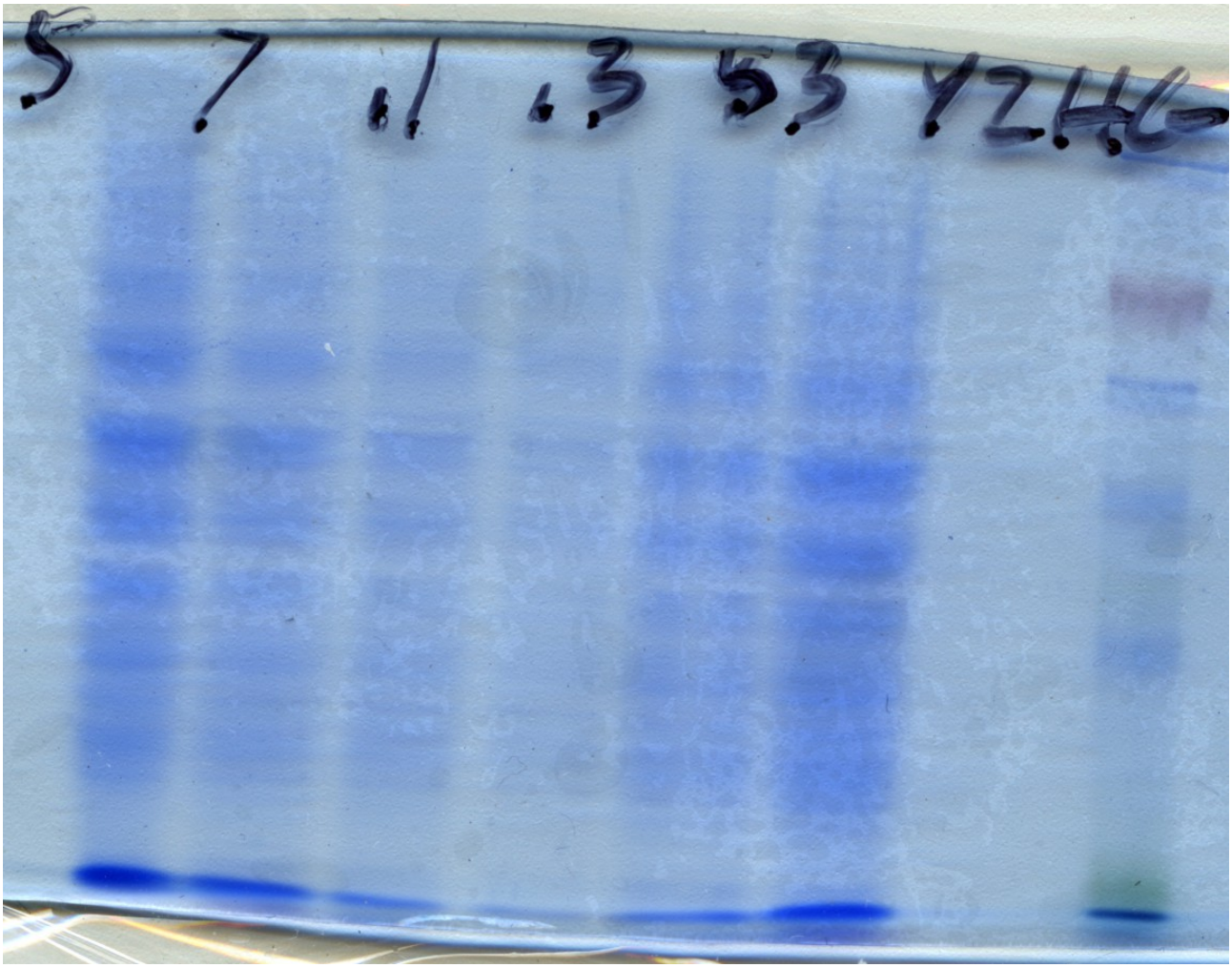
GGCTATCATACATATGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAATTCCACCCAAGCAGTGGTATC
AACGCAGAGTGGCCATTATGGCCGGGGCACAAGAAGAAGGTTGAAGCCAAGAACGCTCTCGAGAACTACGCTTACA
ACATGAGGAACACCATCCAAGACGAGAAGATTGGTGAGAAGCTCCCGGCTGCAGACAAGAAGAAGATCGAGGATTC
TATTGAGCAGGCGATTCAATGGCTCGAGGGTAACCAGTTGGCTGAGGCTGATGAGTTCGAAGACAAGATGAAGGAA
TTGGAGAGCATCTGCAACCCAATCATTGCCAAGATGTACCAAGGAGCTGGTGGTGAAGCCGGTGGTCCAGGTGCCT
CTGGTATGGACGATGATGCTCCCCTGCTTCAGGCGGTGCTGGACCTAAGATCGAGGAGGTCGACTAATTTGTTGGA
CATTGACCTCTCTCTTTCTCCTATCTCTATCTCTTTTACTTGCTTTTTTTGATCTGTTAAGACTTTTTATGTTGGGCTTTT
TTAAAGAAGCCCATTTTGTGGTGTTTTTTGGTTAGTACTATTTTGAACAATGGTTGGTTCTATACCAGTTTAGCTACGAT
GACGGATAAAATTAAAAGTTTGCCATTTTCTCTCATATATAAAAAAAAAAAAAAAAAAAAAAAAACTTGGCCCCCCCCC
CCCCCCCTAGGGGGGGGGGTTTAATCGGGACCCCCCGGCCGGGGGGGAAAATATCCTTATATTTATA

All sequence information can be downloaded here:

https://docs.google.com/file/d/0B1NgzredCy7YMGhBNlRpUkpzUTQ/edit?usp=sharing

5.3: Western Blot – Coomasie stained blot

Below is an image of the coomasie stained western blot mentioned in 2.4.8.



6: Bibliography

1. Bernoux, M., Ellis, J. G. & Dodds, P. N. New insights in plant immunity signaling activation. *Current Opinion in Plant Biology* **14,** 512–8 (2011).

2. Zipfel, C. *et al.* Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* **428,** 764–7 (2004).

3. Asai, T. *et al.* MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415,** 977–83 (2002).

4. Zipfel, C. *et al.* Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* **125,** 749–60 (2006).

5. Greeff, C., Roux, M., Mundy, J. & Petersen, M. Receptor-like kinase complexes in plant innate immunity. *Frontiers in Plant Science* **3,** 209 (2012).

6. Mukhtar, M. S. *et al.* Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* **333,** 596–601 (2011).

7. Demianski, A. J., Chung, K. M. & Kunkel, B. N. Analysis of Arabidopsis JAZ gene expression during Pseudomonas syringae pathogenesis. *Molecular Plant Pathology* **13,** 46–57 (2012).

8. Brooks, D. M., Bender, C. L. & Kunkel, B. N. The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid dependent defences in Arabidopsis thaliana. *Molecular Plant Pathology* **6,** 629–39 (2005).

9. Zheng, X.-Y. *et al.* Coronatine promotes Pseudomonas syringae virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host & Microbe* **11,** 587–96 (2012).

10. Elmore, J. M., Lin, Z.-J. D. & Coaker, G. Plant NB-LRR signaling: upstreams and downstreams. *Current Opinion in Plant Biology* **14,** 365–71 (2011).

11. Krasileva, K. V, Dahlbeck, D. & Staskawicz, B. J. Activation of an Arabidopsis resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. *The Plant Cell* **22,** 2444–58 (2010).

12. Jones, J. D. G. & Dangl, J. L. The plant immune system. *Nature* **444,** 323–9 (2006).

13. Cui, J. *et al.* Pseudomonas syringae manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 1791–6 (2005).

14. Feys, B. J. & Parker, J. E. Interplay of signaling pathways in plant disease resistance. *Trends in Genetics* **16,** 449–55 (2000).

15. Li, J., Brader, G. & Palva, E. T. The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *The Plant Cell* **16,** 319–31 (2004).

16. Wager, A. & Browse, J. Social Network: JAZ Protein Interactions Expand Our Knowledge of Jasmonate Signaling. *Frontiers in Plant Science* **3,** 41 (2012).

17. Fonseca, S., Chico, J. M. & Solano, R. The jasmonate pathway: the ligand, the

receptor and the core signalling module. *Current Opinion in Plant Biology* **12,** 539–47 (2009).

18. Chung, H. S., Niu, Y., Browse, J. & Howe, G. A. Top hits in contemporary JAZ: an update on jasmonate signaling. *Phytochemistry* **70,** 1547–59 (2009).

19. Acosta, I. F. & Farmer, E. E. Jasmonates. *The Arabidopsis book/American Society of Plant Biologists* **8,** e0129 (2010).

20. Pauwels, L. & Goossens, A. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. *The Plant Cell* **23,** 3089–100 (2011).

21. Kombrink, E. Chemical and genetic exploration of jasmonate biosynthesis and signaling paths. *Planta* **236,** 1351–66 (2012).

22. Browse, J. Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual Review of Plant Biology* **60,** 183–205 (2009).

23. Schaller, A. & Stintzi, A. Enzymes in jasmonate biosynthesis - structure, function, regulation. *Phytochemistry* **70,** 1532–8 (2009).

24. Suza, W. P. & Staswick, P. E. The role of JAR1 in Jasmonoyl-L-isoleucine production during Arabidopsis wound response. *Planta* **227,** 1221–32 (2008).

25. Thines, B. *et al.* JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* **448,** 661–5 (2007).

26. Vanholme, B., Grunewald, W., Bateman, A., Kohchi, T. & Gheysen, G. The TIFY family previously known as ZIM. *Trends in Plant Science* **12,** 239–44 (2007).

27. Chung, H. S. *et al.* Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling. *The Plant Journal* **63,** 613–22 (2010).

28. Chini, a *et al.* The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448,** 666–71 (2007).

29. Chung, H. S. & Howe, G. A. A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. *The Plant Cell* **21,** 131–45 (2009).

30. Kazan, K. & Manners, J. M. JAZ repressors and the orchestration of phytohormone crosstalk. *Trends in Plant Science* **17,** 22–31 (2012).

31. Pauwels, L. *et al.* NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* **464,** 788–91 (2010).

32. Chini, A., Fonseca, S., Chico, J. M., Fernández-Calvo, P. & Solano, R. The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. *The Plant Journal* **59,** 77–87 (2009).

33. Fonseca, S. *et al.* (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nature Chemical Biology* **5,** 344–50 (2009).

34. Grunewald, W. *et al.* Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. *EMBO Reports* **10,** 923–8 (2009).

35. Sheard, L. B. *et al.* Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature* **468,** 400–5 (2010).

36. Shyu, C. *et al.* JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in Arabidopsis. *The Plant Cell*

24, 536–50 (2012).

37. Melotto, M. *et al.* A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. *The Plant Journal* **55,** 979–88 (2008).

38. Causier, B., Ashworth, M., Guo, W. & Davies, B. The TOPLESS interactome: a framework for gene repression in Arabidopsis. *Plant Physiology* **158,** 423–38 (2012).

39. Kagale, S., Links, M. G. & Rozwadowski, K. Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. *Plant Physiology* **152,** 1109–34 (2010).

40. Consortium, A. I. M. Evidence for network evolution in an Arabidopsis interactome map. *Science* **333,** 601–7 (2011).

41. Van den Burg, H. a. & Takken, F. L. W. SUMO-, MAPK-, and resistance protein-signaling converge at transcription complexes that regulate plant innate immunity. *Plant Signaling & Behavior* **5,** 1597–1601 (2010).

42. Kim, K.-C., Lai, Z., Fan, B. & Chen, Z. Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *The Plant Cell* **20,** 2357–71 (2008).

43. Zhu, Z. *et al.* Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 12539–44 (2011).

44. Lorenzo, O. & Chico, J. JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *The Plant Cell* **16,** 1938–50 (2004).

45. Fernández-Calvo, P. *et al.* The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *The Plant Cell* **23,** 701–15 (2011).

46. Qi, T. *et al.* The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. *The Plant Cell* **23,** 1795–814 (2011).

47. Solfanelli, C., Poggi, A., Loreti, E., Alpi, A. & Perata, P. Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. *Plant Physiology* **140,** 637–46 (2006).

48. Tian, D., Tooker, J., Peiffer, M., Chung, S. H. & Felton, G. W. Role of trichomes in defense against herbivores: comparison of herbivore response to woolly and hairless trichome mutants in tomato (Solanum lycopersicum). *Planta* **236,** 1053–66 (2012).

49. Niu, Y., Figueroa, P. & Browse, J. Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. *Journal of Experimental Botany* **62,** 2143–54 (2011).

50. Staswick, P. E. JAZing up jasmonate signaling. *Trends in Plant Science* **13,** 66–71 (2008).

51. Mosblech, A., Thurow, C., Gatz, C., Feussner, I. & Heilmann, I. Jasmonic acid perception by COI1 involves inositol polyphosphates in Arabidopsis thaliana. *The Plant Journal* **65,** 949–57 (2011).

52. Koo, A. J. K., Cooke, T. F. & Howe, G. A. Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 9298–303 (2011).

53. Heitz, T. *et al.* Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone Jasmonoyl-isoleucine for catabolic turnover. *The Journal of Biological Chemistry* **287,** 6296–306 (2012).

54. Koo, A. J. K. & Howe, G. A. Catabolism and deactivation of the lipid-derived hormone jasmonoyl-isoleucine. *Frontiers in Plant Science* **3,** 19 (2012).

55. Katsir, L., Schilmiller, A. L., Staswick, P. E., He, S. Y. & Howe, G. A. COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 7100–5 (2008).

56. Chung, H. S. *et al.* Regulation and function of Arabidopsis JASMONATE ZIM-domain genes in response to wounding and herbivory. *Plant Physiology* **146,** 952–64 (2008).

57. Wang, K. L.-C., Li, H. & Ecker, J. R. Ethylene biosynthesis and signaling networks. *The Plant Cell* **Suppl 1,** S131–51 (2002).

58. Shan, X., Yan, J. & Xie, D. Comparison of phytohormone signaling mechanisms. *Current Opinion in Plant Biology* **15,** 84–91 (2012).

59. Qiao, H., Chang, K. N., Yazaki, J. & Ecker, J. R. Interplay between ethylene, ETP1/ETP2 F-box proteins, and degradation of EIN2 triggers ethylene responses in Arabidopsis. *Genes & Development* **23,** 512–21 (2009).

60. Gagne, J. M. *et al.* Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 6803–8 (2004).

61. Verhage, A., van Wees, S. C. M. & Pieterse, C. M. J. Plant immunity: it's the hormones talking, but what do they say? *Plant Physiology* **154,** 536–40 (2010).

62. Schenk, P. M. *et al.* Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 11655–60 (2000).

63. Moffat, C. S. *et al.* ERF5 and ERF6 play redundant roles as positive regulators of JA/Et-mediated defense against Botrytis cinerea in Arabidopsis. *PloS one* **7,** e35995 (2012).

64. Zhou, C., Zhang, L., Duan, J., Miki, B. & Wu, K. HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. *The Plant Cell* **17,** 1196–204 (2005).

65. An, F. *et al.* Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and

EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in Arabidopsis. *The Plant Cell* **22,** 2384–401 (2010).

66. Hou, X., Ding, L. & Yu, H. Crosstalk between GA and JA signaling mediates plant growth and defense. *Plant Cell Reports* **32,** 1067–1074 (2013).

67. Hedden, P. & Phillips, A. L. Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science* **5,** 523–30 (2000).

68. Rieu, I. *et al.* The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *The Plant Journal* **53,** 488–504 (2008).

69. Yang, D. L. *et al.* Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proceedings of the National Academy of Sciences of the United States of America* **109,** E1192–200 (2012).

70. Hou, X., Lee, L. Y. C., Xia, K., Yan, Y. & Yu, H. DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Developmental Cell* **19,** 884–94 (2010).

71. Yamaguchi, S. Gibberellin Biosynthesis in Arabidopsis. *Phytochemistry Reviews* **5,** 39–47 (2006).

72. MacMillan, J. & Takahashi, N. Proposed procedure for the allocation of trivial names to the gibberellins. *Nature* **217,** 170–171 (1968).

73. Gao, X. H., Xiao, S. L., Yao, Q. F., Wang, Y. J. & Fu, X. D. An updated GA signaling "relief of repression" regulatory model. *Molecular Plant* **4,** 601–6 (2011).

74. Feng, S. *et al.* Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature* **451,** 475–9 (2008).

75. De Lucas, M. *et al.* A molecular framework for light and gibberellin control of cell elongation. *Nature* **451,** 480–4 (2008).

76. Leivar, P. & Quail, P. H. PIFs: pivotal components in a cellular signaling hub. *Trends in Plant Science* **16,** 19–28 (2011).

77. Cheng, H. *et al.* Gibberellin acts through jasmonate to control the expression of MYB21, MYB24, and MYB57 to promote stamen filament growth in Arabidopsis. *PLoS Genetics* **5,** (2009).

78. Hong, G. J., Xue, X. Y., Mao, Y. B., Wang, L. J. & Chen, X. Y. Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. *The Plant Cell* **24,** 2635–48 (2012).

79. Wild, M. *et al.* The Arabidopsis DELLA RGA-LIKE3 is a direct target of MYC2 and modulates jasmonate signaling responses. *The Plant Cell* **24,** 3307–19 (2012).

80. Navarro, L. *et al.* DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Current Biology* **18,** 650–655 (2008).

81. Robert-Seilaniantz, A., Grant, M. & Jones, J. D. G. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology* **49,** 317–43 (2011).

82. Nambara, E. & Marion-Poll, A. Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56,** 165–85 (2005).

83. Raghavendra, A. S., Gonugunta, V. K., Christmann, A. & Grill, E. ABA perception and signalling. *Trends in Plant Science* **15,** 395–401 (2010).

84. De Torres-Zabala, M. *et al.* Pseudomonas syringae pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. *The EMBO journal* **26,** 1434–43 (2007).

85. Cutler, A. & Krochko, J. Formation and breakdown of ABA. *Trends in Plant Science* **4,** 472–478 (1999).

86. Seo, M. *et al.* The Arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 12908–13 (2000).

87. Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R. & Abrams, S. R. Abscisic acid: emergence of a core signaling network. *Annual Review of Plant Biology* **61,** 651–79 (2010).

88. Shang, Y. *et al.* The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *The Plant Cell* **22,** 1909–35 (2010).

89. Mohr, P. G. & Cahill, D. M. Abscisic acid influences the susceptibility of Arabidopsis thaliana to Pseudomonas syringae pv. tomato and Peronospora parasitica. *Functional Plant Biology* **30,** 461 (2003).

90. Adie, B. A. T. *et al.* ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *The Plant Cell* **19,** 1665–81 (2007).

91. Lackman, P. *et al.* Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 5891–6 (2011).

92. Zeng, W. *et al.* A genetic screen reveals Arabidopsis stomatal and/or apoplastic defenses against Pseudomonas syringae pv. tomato DC3000. *PLoS Pathogens* **7,** e1002291 (2011).

93. Melotto, M., Underwood, W., Koczan, J., Nomura, K. & He, S. Y. Plant stomata function in innate immunity against bacterial invasion. *Cell* **126,** 969–80 (2006).

94. Kim, T. H. Plant stress surveillance monitored by ABA and disease signaling interactions. *Molecules and Cells* **33,** 1–7 (2012).

95. Dempsey, D. A., Vlot, A. C., Wildermuth, M. C. & Klessig, D. F. Salicylic Acid biosynthesis and metabolism. *The Arabidopsis book/American Society of Plant Biologists* **9,** (2011).

96. Fu, Z. Q. *et al.* NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* **486,** 228–32 (2012).

97. Attaran, E. & He, S. Y. The long-sought-after salicylic Acid receptors. *Molecular Plant* **5,** 971–3 (2012).

98. Tada, Y. *et al.* Plant immunity requires conformational changes of NPR1 via S-

nitrosylation and thioredoxins. *Science* **321,** 952–6 (2008).

99. Loake, G. & Grant, M. Salicylic acid in plant defence--the players and protagonists. *Current Opinion in Plant Biology* **10,** 466–72 (2007).

100. Gust, A. A. & Nürnberger, T. Plant immunology: A life or death switch. *Nature* **486,** 198–9 (2012).

101. Kazan, K. & Manners, J. M. Jasmonate signaling: toward an integrated view. *Plant Physiology* **146,** 1459–68 (2008).

102. Mur, L., Kenton, P. & Atzorn, R. Outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology* **140,** 249–62 (2006).

103. Ndamukong, I. *et al.* SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. *The Plant Journal* **50,** 128–39 (2007).

104. DeFraia, C. & Mou, Z. The role of the Elongator complex in plants. *Plant Signaling & Behavior* **6,** 19–22 (2011).

105. Fu, Z. Q. *et al.* NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* **486,** 228–32 (Supplementary Materials) (2012).

106. Choi, S.-M. *et al.* HDA19 is required for the repression of salicylic acid biosynthesis and salicylic acid-mediated defense responses in Arabidopsis. *The Plant Journal* **71,** 135–46 (2012).

107. Vlot, A. C., Dempsey, D. A. & Klessig, D. F. Salicylic Acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology* **47,** 177–206 (2009).

108. Spoel, S. H., Johnson, J. S. & Dong, X. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 18842–7 (2007).

109. Leon-Reyes, A. *et al.* Ethylene signaling renders the jasmonate response of Arabidopsis insensitive to future suppression by salicylic Acid. *Molecular Plant-Microbe Interactions* **23,** 187–97 (2010).

110. Van Der Does, D. *et al.* Salicylic Acid Suppresses Jasmonic Acid Signaling Downstream of SCFCOI1-JAZ by Targeting GCC Promoter Motifs via Transcription Factor ORA59. *The Plant Cell* **25,** 744–761 (2013).

111. Chen, H. *et al.* ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in Arabidopsis. *The Plant Cell* **21,** 2527–40 (2009).

112. Atilio J., B. & Causin, H. F. The central role of amino acids on nitrogen utilization and plant growth. *Journal of Plant Physiology* **149,** 358–362 (1996).

113. Sønderby, I. E., Geu-Flores, F. & Halkier, B. A. Biosynthesis of glucosinolates - gene discovery and beyond. *Trends in Plant Science* **15,** 283–90 (2010).

114. Stuttmann, J. *et al.* Perturbation of Arabidopsis amino acid metabolism causes incompatibility with the adapted biotrophic pathogen Hyaloperonospora arabidopsidis. *The Plant Cell* **23,** 2788–803 (2011).

115. Liu, G. *et al.* Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. *The Plant Cell* **22,** 3845–63 (2010).

116. Návarová, H., Bernsdorff, F., Döring, A.-C. & Zeier, J. Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *The Plant Cell* **24,** 5123–41 (2012).

117. Renault, H. *et al.* The Arabidopsis pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance. *BMC Plant Biology* **10,** 20 (2010).

118. Renault, H. *et al.* GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in Arabidopsis thaliana. *Plant & Cell Physiology* **52,** 894–908 (2011).

119. Fait, A., Fromm, H., Walter, D., Galili, G. & Fernie, A. R. Highway or byway: the metabolic role of the GABA shunt in plants. *Trends in Plant Science* **13,** 14–9 (2008).

120. Michaeli, S. *et al.* A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. *The Plant Journal* **67,** 485–98 (2011).

121. Bouché, N. & Fromm, H. GABA in plants: just a metabolite? *Trends in Plant Science* **9,** 110–5 (2004).

122. Bouché, N., Fait, A., Zik, M. & Fromm, H. The root-specific glutamate decarboxylase (GAD1) is essential for sustaining GABA levels in Arabidopsis. *Plant Molecular Biology* **55,** 315–25 (2004).

123. Zik, M., Arazi, T., Snedden, W. a & Fromm, H. Two isoforms of glutamate decarboxylase in Arabidopsis are regulated by calcium/calmodulin and differ in organ distribution. *Plant Molecular Biology* **37,** 967–75 (1998).

124. Shelp, B. J., Mullen, R. T. & Waller, J. C. Compartmentation of GABA metabolism raises intriguing questions. *Trends in Plant Science* **17,** 57–9 (2012).

125. Szabados, L. & Savouré, A. Proline: a multifunctional amino acid. *Trends in Plant Science* **15,** 89–97 (2010).

126. Meyer, A., Eskandari, S., Grallath, S. & Rentsch, D. AtGAT1, a high affinity transporter for gamma-aminobutyric acid in Arabidopsis thaliana. *The Journal of Biological Chemistry* **281,** 7197–204 (2006).

127. Grallath, S., Weimar, T. & Meyer, A. The AtProT family. Compatible solute transporters with similar substrate specificity but differential expression patterns. *Plant Physiology* **137,** 117–26 (2005).

128. Lee, Y. H. & Tegeder, M. Selective expression of a novel high-affinity transport system for acidic and neutral amino acids in the tapetum cells of Arabidopsis flowers. *The Plant Journal* **40,** 60–74 (2004).

129. Ward, J. L. *et al.* The metabolic transition during disease following infection of Arabidopsis thaliana by Pseudomonas syringae pv. tomato. *The Plant Journal* 443–457 (2010). doi:10.1111/j.1365-313X.2010.04254.x

130. Rico, A. & Preston, G. M. Pseudomonas syringae pv. tomato DC3000 uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. *Molecular Plant-Microbe Interactions* **21,** 269–82 (2008).

131. Park, D. H. *et al.* Mutations in γ-aminobutyric acid (GABA) transaminase genes in plants or Pseudomonas syringae reduce bacterial virulence. *The Plant Journal* **64,** 318–30 (2010).

132. Zeier, J. New insights into the regulation of plant immunity by amino acid metabolic pathways. *Plant, Cell & Environment* (2013). doi:10.1111/pce.12122

133. Verslues, P. & Sharma, S. Proline metabolism and its implications for plant-environment interaction. *The Arabidopsis Book/American Society of Plant Biologists* **8,** e0140 (2010).

134. Székely, G. *et al.* Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. *The Plant Journal* **53,** 11–28 (2008).

135. Lehmann, S., Funck, D., Szabados, L. & Rentsch, D. Proline metabolism and transport in plant development. *Amino Acids* **39,** 949–62 (2010).

136. Tegeder, M. Transporters for amino acids in plant cells: some functions and many unknowns. *Current Opinion in Plant Biology* **15,** 315–21 (2012).

137. Fabro, G., Kovács, I., Pavet, V., Szabados, L. & Alvarez, M. E. Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. *Molecular Plant-Microbe Interactions* **17,** 343–50 (2004).

138. Strizhov, N. *et al.* Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. *The Plant Journal* **12,** 557–69 (1997).

139. Cecchini, N. M., Monteoliva, M. I. & Alvarez, M. E. Proline dehydrogenase contributes to pathogen defense in Arabidopsis. *Plant Physiology* **155,** 1947–59 (2011).

140. Senthil-Kumar, M. & Mysore, K. S. Ornithine-delta-aminotransferase and proline dehydrogenase genes play a role in non-host disease resistance by regulating pyrroline-5-carboxylate metabolism-induced hypersensitive response. *Plant, Cell & Environment* **35,** 1329–43 (2012).

141. Haudecoeur, E. *et al.* Proline antagonizes GABA-induced quenching of quorum-sensing in Agrobacterium tumefaciens. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 14587–92 (2009).

142. Deuschle, K. *et al.* The role of [Delta]1-pyrroline-5-carboxylate dehydrogenase in proline degradation. *The Plant Cell* **16,** 3413–25 (2004).

143. Miller, G. *et al.* Unraveling delta 1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. *The Journal of Biological Chemistry* **284,** 26482–92 (2009).

144. Buell, C. R. *et al.* The complete genome sequence of the Arabidopsis and tomato pathogen Pseudomonas syringae pv. tomato DC3000. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 10181–6 (2003).

145. Lindeberg, M., Cunnac, S. & Collmer, A. Pseudomonas syringae type III effector repertoires: last words in endless arguments. *Trends in Microbiology* **20,** 199–208 (2012).

146. Bull, C. T. *et al.* Multilocus sequence typing of Pseudomonas syringae sensu lato confirms previously described genomospecies and permits rapid identification of P. syringae pv. coriandricola and P. syringae pv. apii causing bacterial leaf spot on parsley. *Phytopathology* **101,** 847–58 (2011).

147. Bender, C. Pseudomonas syringae phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiology and Molecular Biology Reviews* **63,** 266–92 (1999).

148. Geng, X., Cheng, J., Gangadharan, A. & Mackey, D. The coronatine toxin of Pseudomonas syringae is a multifunctional suppressor of Arabidopsis defense. *The Plant Cell* **24,** 4763–74 (2012).

149. Bu, Q. *et al.* Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell Research* **18,** 756–67 (2008).

150. Choquer, M. *et al.* Botrytis cinerea virulence factors: new insights into a necrotrophic and polyphageous pathogen. *FEMS Microbiology Letters* **277,** 1–10 (2007).

151. Rossi, F. R. *et al.* The sesquiterpene botrydial produced by Botrytis cinerea induces the hypersensitive response on plant tissues and its action is modulated by salicylic acid and jasmonic acid signaling. *Molecular Plant-Microbe Interactions* **24,** 888–96 (2011).

152. Frías, M., Brito, N. & González, C. The Botrytis cinerea cerato-platanin BcSpl1 is a potent inducer of systemic acquired resistance (SAR) in tobacco and generates a wave of salicylic acid expanding. *Molecular Plant Pathology* 1–6 (2012). doi:10.1111/j.1364-3703.2012.00842.x

153. El Oirdi, M. *et al.* Botrytis cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell* **23,** 2405–21 (2011).

154. Frías, M., González, C. & Brito, N. BcSpl1, a cerato-platanin family protein, contributes to Botrytis cinerea virulence and elicits the hypersensitive response in the host. *The New Phytologist* **192,** 483–95 (2011).

155. Govrin, E. M. & Levine, A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. *Current Biology* **10,** 751–7 (2000).

156. De Torres, M. *et al.* Pseudomonas syringae effector AvrPtoB suppresses basal defence in Arabidopsis. *The Plant Journal* **47,** 368–82 (2006).

157. King, E. O., Ward, M. K. & Raney, D. E. Two simple media for the demonstration of pyocyanin and fluorescin. *The Journal of Laboratory and Clinical Medicine* **44,** 301–7 (1954).

158. Bertani, G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *Journal of Bacteriology* **62,** 293–300 (1951).

159. Langlois-Meurinne, M., Gachon, C. M. M. & Saindrenan, P. Pathogen-responsive expression of glycosyltransferase genes UGT73B3 and UGT73B5 is necessary for resistance to Pseudomonas syringae pv tomato in Arabidopsis. *Plant Physiology* **139,** 1890–901 (2005).

160. Shorty buffer stock solution (5X). *Cold Spring Harbor Protocols* **2009,**

pdb.rec11660–pdb.rec11660 (2009).

161. Forcat, S., Bennett, M. H., Mansfield, J. W. & Grant, M. R. A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. *Plant methods* **4,** 1–8 (2008).

162. Printen, J. & Sprague-Jr, G. Protein-protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade. *Genetics* **138,** 609–19 (1994).

163. BIO RAD. Transfer Buffer Formulations. at <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6211.pdf>

164. Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7,** 203–14 (2000).

165. The Arabidopsis Information Resource (TAIR). at <www.arabidopsis.org>

166. Guo, J. *et al.* Proteomic identification of MYC2-dependent jasmonate-regulated proteins in Arabidopsis thaliana. *Proteome Science* **10,** 57 (2012).

167. Glombitza, S. *et al.* Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. *Plant Molecular Biology* **54,** 817–35 (2004).

168. Pauwels, L. *et al.* Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 1380–5 (2008).

169. Feng, F. & Zhou, J. M. Plant-bacterial pathogen interactions mediated by type III effectors. *Current Opinion in Plant Biology* **15,** 469–76 (2012).

170. Ryu, H. Y. *et al.* Modulations of AtGSTF10 expression induce stress tolerance and BAK1-mediated cell death. *Biochemical and Biophysical Research Communications* **379,** 417–22 (2009).

171. Meldau, S., Erb, M. & Baldwin, I. T. Defence on demand: mechanisms behind optimal defence patterns. *Annals of Botany* **110,** 1503–14 (2012).

172. Tian, M. *et al.* The combined use of photoaffinity labeling and surface plasmon resonance-based technology identifies multiple salicylic acid-binding proteins. *The Plant Journal* **72,** 1027–1038 (2012).

173. Mou, Z., Fan, W. & Dong, X. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113,** 935–44 (2003).

174. Clément, M. *et al.* The cytosolic/nuclear HSC70 and HSP90 molecular chaperones are important for stomatal closure and modulate abscisic acid-dependent physiological responses in Arabidopsis. *Plant Physiology* **156,** 1481–92 (2011).

175. Noël, L. D. *et al.* Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. *The Plant Cell* **19,** 4061–76 (2007).

176. Cazalé, A. C. *et al.* Altered expression of cytosolic/nuclear HSC70-1 molecular chaperone affects development and abiotic stress tolerance in Arabidopsis thaliana. *Journal of Experimental Botany* **60,** 2653–64 (2009).

177. Jelenska, J., Van Hal, J. A. & Greenberg, J. T. Pseudomonas syringae hijacks plant

stress chaperone machinery for virulence. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 13177–82 (2010).

178. Uppalapati, S. R. *et al.* SGT1 contributes to coronatine signaling and Pseudomonas syringae pv. tomato disease symptom development in tomato and Arabidopsis. *The New Phytologist* **189,** 83–93 (2011).

179. Cerrudo, I. *et al.* Low red/far-red ratios reduce Arabidopsis resistance to Botrytis cinerea and jasmonate responses via a COI1-JAZ10-dependent, salicylic acid-independent mechanism. *Plant Physiology* **158,** 2042–52 (2012).

180. Wallace, W., Secor, J. & Schrader, L. E. Rapid Accumulation of gamma-Aminobutyric Acid and Alanine in Soybean Leaves in Response to an Abrupt Transfer to Lower Temperature, Darkness, or Mechanical Manipulation. *Plant Physiology* **75,** 170–5 (1984).

181. Bown, A. W., Macgregor, K. B. & Shelp, B. J. Gamma-aminobutyrate: defense against invertebrate pests? *Trends in Plant Science* **11,** 424–7 (2006).

182. Shelp, B. J., Bown, A. W. & Faure, D. Extracellular gamma-aminobutyrate mediates communication between plants and other organisms. *Plant Physiology* **142,** 1350–2 (2006).